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# THE NUTRITION OF INVERTEBRATES

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A science of nutrition, as McCay (125, 127) has emphasized, should rest on a knowledge of the nutritional needs of many different types of organisms. The nutrition of bacteria (104) and of vertebrates has been extensively investigated. Among the invertebrates, only the protozoa and insects have been studied to any extent with respect to their nutritional requirements. This review will summarize the considerable information which has accumulated since the subjects of protozoan and insect nutrition were last completely reviewed. It will also bring together what little is known concerning the nutritional requirements of other invertebrates.

By nutritional requirements is meant, not necessarily the natural food of the organism, but the fundamental chemical substances which the organism must get preformed from its environment in order to live, grow, and reproduce. For example, garbage may serve as a suitable food for rats, but it is not one of their nutritional requirements, in the sense in which riboflavin and certain amino-acids are requirements. One need only refer to the *Compendium of Culture Methods* (55) to see at once that a great deal more is known about the food of invertebrates than about their nutritional requirements. A study of the latter frequently involves special methods, such as rearing the organism in absolutely pure culture free from all other living organisms, including microscopic forms.

The invertebrates, like all other organisms, must somehow obtain energy, carbon, nitrogen, sulfur, phosphorus, and the mineral elements essential for the functioning of protoplasm. All the metazoa and protozoa thus far studied, other than certain of the free-living colorless flagellates, appear to require both their nitrogen and carbon already combined in organic compounds. They do not all require protein,

fats, and carbohydrates, and frequently proteins alone or their split products can serve as the nitrogen and carbon source. Recent work indicates that probably all of the metazoa and most of the protozoa require in addition accessory growth factors (i.e., substances which do not act as major sources of energy or building material but which are essential to the normal life of the organism, and which the organism is incapable of synthesizing) (cf. 104). Any detailed study of the nitrogen and carbon sources of an organism, like that of Rose (158) with rats, must be preceded by at least a partial purification of the accessory growth factors. Hence it is not surprising that much of the work to be reviewed has been concerned with the requirements for accessory factors, rather than with, for example, the protein or carbohydrate requirements.

I. THE PROTOZOA. *A. Free-living plant-like green and colorless flagellates.* This group of organisms, with affinities for the algae on the one hand and for the true protozoa on the other, shows all the possible gradations in complexity of nutritional requirements, from forms capable of autotrophic nutrition in light to forms requiring special growth factors. Except for the possession of pigment, the colorless flagellates of this group have their exact morphological counterparts among the chlorophyll-bearing species. From a physiological standpoint, however, it is very convenient to create a special group for the colorless forms. Lwoff (113, 115) designated them as leucophytes—protists having a plast but no chlorophyll and synthesizing starch or paramylum as their carbohydrate reserves,—and the green species as chlorophytes.

Since most of the green flagellates which have been studied have been found to be capable of autotrophic nutrition in light, in the same way as the green plants, the present discussion will be confined to results with the leucophytes and with chlorophytes grown in the dark.

1. *Carbon nutrition.* Pringsheim (152) first showed the greatly stimulating effect of acetate on pure cultures of *Polytoma uvella.* This effect of acetic acid and certain related compounds has since been demonstrated for all species of leucophytes and chlorophytes grown in the dark which have been investigated (115). The degree of stimulation varies greatly with the species concerned and the environmental conditions. For some species it is so great that growth in a peptone medium devoid of acetate is almost nil.

The effects of different organic acids have been observed by Lwoff and collaborators, Pringsheim, Loefer, and others, and have been re-

cently summarized by Provasoli (153), whose own work has contributed much valuable information. As is evident from table 1, acetic acid is the only organic acid which stimulated the growth of all species. For some species, however, acids up to and including myristic were active. If a species was able to utilize an alcohol, growth was about the same

TABLE 1

*The growth response of various plant-like flagellates to organic acids and alcohols. (From Provasoli, 1938)*

| | CHLOROGONIUM EUCHLORUM | HYALOGONIUM KLEBSII | POLYTOMA OBTUSUM | CHLOROGONIUM ELONGATUM | CHLAMYDOMONAS AGLOEFORMIS | HAEMATOCOCCUS PLUVIALIS | POLYTOMA CAUDATUM | POLYTOMA UVELLA | ASTASIA QUARTANA | POLYTOMA OCELLATUM | POLYTOMELLA COECA | CHILOMONAS PARAMOECIUM | EUGLENA GRACILIS | EUGLENA GRACILIS VAR. UROPHORA | ASTASIA CHATTONI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Acid, acetic | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acid, propionic | − | − | − | − | − | − | − | − | + | + | + | − | + | + | + |
| Acid, butyric | − | − | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Acid, isobutyric | − | − | − | − | − | − | − | − | ± | + | − | − | − | + | ± |
| Acid, valeric | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + |
| Acid, isovaleric | | | − | | | | − | − | ± | ± | − | ± | − | − | − |
| Acid, capraic | − | − | − | − | − | − | − | + | + | + | + | + | + | + | + |
| Acid, isocaproic | | | − | | | | − | + | + | + | ± | + | ± | ± | ± |
| Acid, heptylic | | | − | | | | − | − | + | + | + | − | − | + | + |
| Acid, octylic | | | − | | | | − | ± | | + | + | + | + | + | + |
| Acid, nonylic | | | − | | | | − | − | | + | + | ± | + | ± | + |
| Acid, decylic | | | − | | | | − | − | | + | + | | + | ± | ± |
| Acid, lauric | | | | | | | | | | + | ± | | + | ± | ± |
| Acid, myristic | | | | | | | | | | ± | − | ± | ± | ± | ± |
| Alcohol, methyl | | | − | | | | − | − | − | − | − | − | − | − | − |
| Alcohol, ethyl | − | − | − | | | | − | − | − | + | + | + | + | + | + |
| Alcohol, propyl | | | − | | | | − | − | − | + | + | ± | + | + | + |
| Alcohol, isopropyl | | | | | | | − | − | − | − | − | − | − | − | − |
| Alcohol, butyl | | | − | | | | − | − | − | + | + | + | + | + | + |
| Alcohol, isobutyl | | | − | | | | − | − | − | + | + | − | − | + | ± |
| Alcohol, amyl | | | − | | | | − | − | − | ± | − | ± | − | − | − |
| Alcohol, esilic | | | | | | | | | | + | + | + | + | + | + |

as with the corresponding acid, indicating a preliminary oxidation of the alcohol to the acid. Provasoli was able to demonstrate valeric acid in the fluid from a culture of *Polytoma ocellatum* grown in the presence of isoamyl alcohol. He suggested that the different species differ in their oxidative powers. In this connection Provasoli reported that

certain strains of *Polytoma obtusum*, which had been kept over a period of years on a peptone medium and had lost their ability to utilize butyric acid, recovered this ability after a prolonged period of adaptation. Flagellates thus adapted to butyrate could be adapted in a similar manner to caproate. These results suggest the formation of adaptive enzymes, instances of which have been previously reported for bacteria (104) but not for protozoa.

2. *Requirements for sulfur, nitrogen and growth factors.* Sulfur utilization has been studied only in the colorless flagellate *Chilomonas paramecium* (136). The fission rate was highest in media containing reduced sulfur ($Na_2S$, glutathione, cysteine, cystine), lower in media with sulfite and lowest in sulfate media. One of the chief functions of the sulfur appears to be in the oxidation of fat.

The nitrogen requirements of these flagellates have been elucidated in considerable detail. In the presence of light, almost all of the chlorophyll-bearing forms can utilize either the nitrate or ammonium radicals. The apparent exceptions, of considerable physiological interest, are all in the group Euglenidae (43). *Euglena gracilis* could use ammonium but not nitrate as its nitrogen source in completely inorganic media. *E. stellata* could use either ammonium or nitrate providing calcium was present. *E. deses*, however, could use neither ammonium nor nitrate but only various amino acids as the nitrogen source. *E. pisciformis*, with apparently still more complex nitrogen requirements, had to be supplied with peptone. Neither of the two latter species could be grown in the dark. It should be noted, however, that a requirement for peptone may indicate the need for a specific growth factor rather than for a complex source of the bulk of the nitrogen (see below). Moreover, it may be possible to grow *E. pisciformis* in an inorganic medium of different composition from any thus far tried. Thus, although Hutner (102) concluded that *E. anabaena* var. *minor* required a complex nitrogen source, Hall (72) was able, by suitably modifying other constituents of the medium, to grow it autotrophically with ammonium nitrate as the sole nitrogen source. Similarly, *E. gracilis* could be readily grown under autotrophic conditions, but only if manganese was present in the medium (76). This subject has been recently reviewed by Hall (73).

Among the colorless flagellates and the green flagellates grown in the dark, some, like *Polytoma uvella*, can use ammonium as the sole nitrogen source. It is interesting to note that *P. uvella* can do so only if a ferric salt is added to the medium. Other species apparently require amino acids or complex peptones. The recent work of Lwoff and Dusi (118)

indicates that peptone may act by furnishing growth factors, notably the pyrimidine and thiazol of thiamin. This discovery has made it possible to grow a number of these flagellates in entirely synthetic media containing ammonium acetate, iron, suitable salts and either the pyrimidine or thiazol of thiamin or both together (116).

Mast and Pace (135) reported that they could grow *Chilomonas paramecium* not only in simple organic media, such as those which support growth of *Polytoma uvella,* but also in a completely inorganic medium. In this medium, ammonium served as the nitrogen source and carbon dioxide (in a ratio of 1 part $CO_2$ to 5 parts of air) as the carbon source. In the inorganic medium, silicate, which stimulated growth in the simple organic medium, was essential for growth. The cultures grew autotrophically in the dark as well as in the light and there was slight evidence for the formation of nitrate. On this basis it was assumed that the energy necessary for the reduction and utilization of carbon dioxide was derived from the oxidation of ammonium to nitrate, as is the case with certain autotrophic bacteria.

The work of Mast and Pace has not been confirmed by other investigators (111, 75). Lwoff and Dusi's (118) recent very careful work seems to show conclusively that *Chilomonas* can grow with ammonium as its nitrogen source and acetate as its carbon source only if both the pyrimidine and thiazol of thiamin are supplied. These results do not eliminate the possibility that *Chilomonas,* if provided with a high carbon dioxide concentration, silicate, and the essential amounts of pyrimidine and thiazol, could obtain the bulk of its carbon requirements from the carbon dioxide. Burrows (19) found that *Chilomonas* could be induced to grow, although with difficulty, in both the simple organic medium and the inorganic medium with high carbon dioxide tension of Mast and Pace. Growth was light, although it apparently could be carried on indefinitely. In spite of the use of very sensitive tests for nitrite and nitrate neither of these substances could be detected in cultures in the inorganic medium. If the flagellates were actually using carbon dioxide as their carbon source, the manner in which they obtained energy for its reduction remains a mystery. Burrows tested his cultures very thoroughly for bacteria, including nitrifying forms, with uniformly negative results. A source of error was his utilization of cotton plugs, which also cannot be used in careful studies with autotrophic bacteria (184). Then, too, it is possible that under his conditions sufficient numbers of flagellates died in each culture to liberate enough organic substance for another light culture.

Elliott (46) found no effect of "pantothenic acid" on the growth of the

chlorophyte *Haematococcus pluvialis.* He did obtain a marked effect with certain of the so-called plant hormones on the growth in light of *Euglena gracilis* (49). Indole-acetic acid accelerated growth at a concentration of 1:10,000,000, as did indole propionic acid, while indole butyric was active down to a concentration of 1:1,000,000. The effects were much greater at pH 5.6 than at pH 7.4. Interestingly enough, corresponding dilutions of these substances had either no effect or an inhibitory effect on the colorless euglenoid *Khawkinea halli.*

*B. Free-living amoebae and ciliates.* Comparatively little work has been done with amoebae and ciliates in absolutely pure culture, a condition necessary to any study of their nutritional needs. Oehler (146, 147) was the pioneer in this field and obtained one species of ciliate and several species of amoebae in pure culture. Glaser and Coria (61) grew *Trichoda pura* in their sterile hay infusion-serum medium. Since then a number of others of the smaller species of ciliates have been reared free from contaminants (see Hetherington, in 55).

The one species of ciliate which could be most easily grown under bacteria-free conditions and which has lent itself best to studies on nutrition is *Glaucoma pyriformis* (synonyms of which are, according to Hetherington, *Saprophilus oviformis* and *Colpidium striatum*). One strain of this ciliate utilized, as judged by acid production (34), levulose, glucose, galactose and maltose, but not mannite, arabinose, xylose, sucrose, lactose, inulin, or starch while another strain (*Colpidium striatum* (47)) utilized starch, levulose, mannose, and dextrose but not galactose, dextrin, inulin, salicin, melezitose, sucrose, lactose, maltose, or mannite. The closely related ciliate *Colpidium campylum* (47) fermented the same carbohydrates as the latter strain of *Glaucoma* and also maltose. The dextrose consumption of *C. campylum* and *Glaucoma pyriformis* cultured in a tryptone salt solution medium has been found to be 2.34 to 3.92 $\times 10^{-7}$ mgm. per organism per hour for *C. campylum* and 0.5 to 3.29 $\times 10^{-7}$ per organism per hour for *G. pyriformis* (112). Glaser and Coria (63) found that *Saprophilus oviformis* (a hot springs strain of *Glaucoma pyriformis*) and *Trichoda pura* fermented glucose and maltose but not lactose or sucrose. If a suspension of cellulose was added to cultures of these two species of ciliates, or of *Chilodon cucullus, Paramecium caudatum,* or *P. multimicronucleatum,* the turbidity was soon cleared away, indicating the digestion of cellulose by the protozoa.

Elliott (48), working with *Glaucoma pyriformis* (*C. striatum*) and *Colpidium campylum,* confirmed Lwoff's (113) conclusion that these

ciliates could not grow with single amino acids or even with mixtures of several amino acids, but required substances present in peptones. These two species could not grow continuously in media consistiog of zein, gliadin, gelatin, or silk peptone, although they did grow in meat peptone (74). Supplementing gliadin with lysine, or gelatin with tryptophane and isoleucine, had no effect. However, the addition of 0.00005 per cent Difco yeast extract permitted continuous growth in gelatin, zein, or gliadin, suggesting a requirement for special growth factors (see below). In the presence of such a small amount of yeast extract, growth in a gliadin medium was considerably enhanced by the addition of lysine.

The culture by Glaser and Coria (62) of *Paramecium caudatum* free from living microörganisms first emphasized the very complex nutritional requirements of certain free-living protozoa. Numerous investigators had grown *Paramecium* in the presence of pure cultures of live bacteria or yeast. But if the ciliates were washed free from the living microörganisms and furnished with corresponding dead microörganisms, no growth occurred. However, if heat-killed yeast was supplemented with a suitable liver extract and a fragment of rabbit kidney aseptically removed from the animal, excellent growth ensued under completely sterile conditions. All three constituents of the medium were essential for continuous cultivation, but the kidney tissue could be replaced by an extract of kidney sterilized by Berkefeld filtration. It is apparent that *Paramecium* requires numerous special growth factors which, in nature, are supplied to it by the living microörganisms always present in its habitat. The chemical nature of these factors has not as yet been investigated.

"Pantothenic acid," a partially purified yeast growth factor, stimulated the growth of *Glaucoma pyriformis* (*Colpidium striatum*) in the pH range of 5.5 to 6.5, while at pH's above 7.0 growth was slightly less with the acid than without it (46). Pantothenic acid used in place of yeast extract in a zein, gliadin, or gelatin medium did not support growth. Hence it is not the essential substance which is lacking in these proteins and in hydrolyzed silk. Lwoff and Lwoff (119) have shown that this essential substance is thiamin. The addition of thiamin to a hydrolyzed silk-glucose medium permitted continuous growth of *G. pyriformis*. There was no growth if thiamin was replaced by either its pyrimidine or its thiazol. Evidently, *Glaucoma* resembles mammals in requiring the intact thiamin molecule. Thiamin could not be replaced by a number of closely related compounds (120).

Elliott (199) has more recently confirmed this result with his strain designated as *Colpidium striatum*. Thiamin, when added to a tryptone medium rendered itself unsuitable for growth by an hour's autoclaving at pH 9.6, permitted optimum growth at concentrations down to 1:10,000,000. Crystalline riboflavin and a vitamin $B_6$ concentrate had no effect on the growth of the ciliates. In media not containing dextrose, pimelic acid at a concentration of $10^{-6}$ grams per ml. stimulated the growth of *Colpidium campylum* (200), but it had no effect in a gelatin dextrose medium in which the growth is always much greater than in dextrose-free media. No evidence was obtained to indicate that pimelic acid was essential for growth. A valuable study by Kidder, Lilly and Claff (201) shows the tremendous effects which type of food may have on the morphology of a protozoon. *Glaucoma vorax* reared on pure cultures of live bacteria had a characteristic tailed shape, which it lost when reared in a sterile peptone-broth medium. When allowed to feed on the related ciliate *Colpidium campylum*, the *G. vorax* increased many times in size, an effect which was not produced by yeast or flagellates, or by dead *Colpidium* or various extracts of them.

Among the free-living amoebae, *Acanthamoeba castellanii* (113) and *Mayorella palestinensis* (154) have been grown free from other organisms. Reich cultured *M. palestinensis*, a soil amoeba, in a medium consisting of a balanced salt solution containing 1 per cent peptone and 1 per cent dextrose. The peptone could be replaced by serum, gelatin, or autolyzed yeast, but not by single amino acids or ammonium salt. Dextrose was indispensable for good growth, although no acid was produced. Levulose, sucrose, and lactose could replace dextrose.

*Acanthamoeba castellanii*, which grows well in meat peptone but not in silk peptone or in meat peptone heated 20 minutes at 120°C. at pH 9.6, grew in the two latter media if either thiamin or its pyrimidine was added (117). The ciliate *Glaucoma pyriformis*, which requires the intact thiamin molecule, developed well in a peptone medium devoid of thiamin but containing its pyrimidine, its thiazol, and a rich culture of *Acanthamoeba*. It is evident that the amoeba can synthesize thiamin from its pyrimidine and its thiazol.

*C. The parasitic protozoa.* 1. *Hemoflagellates.* A thorough understanding of the nutritional requirements of parasitic protozoa can be obtained only if the organisms are grown in pure culture. This fact explains why more progress has been made with the hemoflagellates than with any other group. Most of these organisms, which occur

principally in the blood of vertebrates or in the alimentary tract of insects, can be readily cultured in media containing blood. The species from vertebrates are already in a practically sterile environment while those from insects can be rather easily freed from bacteria.

The carbohydrate metabolism of the hemoflagellates has attracted considerable attention, especially since Yorke, Adams and Murgatroyd (196) first showed that the pathogenic trypanosomes, except *Trypanosoma cruzi*, require glucose and utilize as much as 7 to 8 mgm. per 1,000 million organisms per hour at 37°C. Since the subject of the metabolism of the pathogenic trypanosomes and its relation to the carbohydrate metabolism of their hosts has been recently reviewed by von Brand (17) it will not be further discussed here.

The other aspect of the nutrition of hemoflagellates which is of great interest is the apparent requirement of many of the species for blood. Cleveland and Collier (31) found that 7 species of *Leptomonas* could be grown in simple glucose-peptone media, while several species of *Leishmania* and *Trypanosoma* gave good growth only in the presence of hemoglobin or hydrolyzed hemoglobin. Lwoff (114) showed that *Strigomonas* (*Leptomonas*) *fasciculata* could grow in peptone solution only in the presence of blood, and that under proper conditions and at blood concentrations of 1:200,000 to 1:500,000 the growth of the flagellates was proportional to the concentration of blood. Blood greatly stimulated the respiration of these protozoa. Lwoff (116) has recently reviewed the literature on the rôle of hematin in the growth of certain hemoflagellates and the hemophilic bacteria. The iron content and the peroxidase activity of hematin are not responsible for its effect, since protoporphyrin, having no iron and no peroxidase activity, affects the respiration and growth of *Strigomonas fasciculatus* just as does hematin, whereas hematohemin, which has both iron and peroxidase activity, has no effect on the flagellate. It seems probable that the hematin or protoporphyrin is used for the synthesis of essential respiratory enzymes. In this connection, it is most interesting that the bacterium *Hemophilus influenzae* does not require hematin if grown anaerobically.

Hematin is not the only growth factor required by the hemoflagellates. *Strigomonas oncopelti*, which does not require hematin and grows in meat peptone but not in hydrolyzed silk, requires the complete thiamin molecule. If this is added, at concentrations as low as $1 \times 10^{-9}$, to a hydrolyzed silk-glucose medium, good and continued growth results (121, 122). Thiamin cannot be replaced by its pyrimidine or its thiazol added either separately or together, or by certain related com-

pounds. *Strigomonas fasciculata* and *S. culicidarum* similarly require thiamin, as well as hemin.

*Schizotrypanum* (*Trypanosoma*) *cruzi*, the most readily cultivated of the pathogenic trypanosomes, has been shown (123) to require ascorbic acid as well as hematin and other as yet unidentified growth factors. This flagellate would not grow in peptone-saline unless a rather large amount of blood was added. It did not grow in peptone with inactivated serum or in peptone with washed or laked blood cells, but did grow in peptone plus serum plus washed blood cells. Peptone with serum and hematin gave no growth. If synthetic l-ascorbic acid was added to the peptone-serum-hemin medium, good growth ensued. All these materials were shown to be essential for growth. *Leishmania tropica* is stated to have the same nutritional requirements as *S. cruzi.*

2. *Intestinal and related flagellates.* Chatton (23) first obtained bacteria-free cultures of an intestinal flagellate, *Trichomastix colubrorum*, in a medium containing blood or a piece of an organ. Cleveland (29) grew the coprophagous organism, *Tritrichomonas fecalis*, in media containing heat-killed bacteria. Witte (185) and Glaser and Coria (64) independently and by different methods obtained pure cultures of *Trichomonas foetus*, the former in a peptone-bouillon-blood medium, the latter in the Locke-egg-blood medium of Kofoid and Wagener (107). At the same time Bos (16) first obtained pure cultures of *Trichomonas columbae*, a parasite of pigeons which invades certain organs of its host. This flagellate could be grown in Locke-egg-serum or in bouillon with liver fragments (autoclaved).

Riedmüller (156) concluded that the growth factors required by *T. foetus* were present in blood serum rather than in the blood cells. Growth was greatly stimulated by glucose, galactose, fructose, lactose, maltose, sucrose, raffinose, dextrin, starch, and glycogen, and with all these carbohydrates a decrease in pH occurred. With pentoses or higher alcohols there was neither growth stimulation nor a decrease in pH. Andrews and von Brand (3) performed quantitative studies on glucose consumption by *T. foetus*.

Cailleau (20) investigated in exhaustive fashion the nutritional requirements of *Eutrichomastix* (*Trichomastix*) *colubrorum*, *Trichomonas columbae* and *T. foetus*. *Eutrichomastix colubrorum* did not grow if whole blood was replaced by rabbit serum, but did grow if the serums of various other species of animals were used, guinea pig and horse serum being best. This flagellate produced much acid with glucose, galactose, levulose, maltose, sucrose, and raffinose, little with lactose, and none

with arabinose, xylose, rhamnose, inulin, dextrin, glycerin, erythrite, sorbite, dulcite, or mannose. *Trichomonas columbae* could be grown in an autoclaved medium containing fragments of veal liver in bouillon in a ratio of about 1:6. In such a medium, a strong acidity was developed with glucose, galactose, maltose, sucrose, dextrin, and soluble starch, little with levulose, lactose, and inulin, and none with arabinose, xylose, rhamnose, raffinose, glycerin, erythrite, sorbite, mannite, or dulcite. Pigeon, rat, or guinea pig serum, but not horse, rabbit, human, or cat serum, supported growth if added to bouillon. Liver extracts prepared with water, alcohol, acetone, or ether could not replace whole liver. The residue left after acetone extraction could not do so, but this residue combined with the acetone extract could. In testing for the nature of the acetone soluble factor, the basic medium used was bouillon with liver which had been extracted with acetone and alcohol. It was soon found that the acetone extract could be replaced by pure cholesterol at dilutions as high as 1:10,000 (when added in alcoholic solution to give a fine suspension in the medium). Cailleau then tested 66 sterols and sterol derivatives as growth factors for *T. columbae* and found certain correlations between chemical structure and activity. For instance, shortening or suppressing the side chain of cholesterol eliminated the activity.

*T. foetus* and *Eutrichomastix colubrorum* also require cholesterol, or other suitable sterols (21, 22). Ascorbic acid is another growth factor required by these flagellates. Thus *E. colubrorum* grew in a medium containing bouillon, egg albumin, cholesterol, ascorbic acid, and peptone, but did not grow if any one of these constituents was omitted (22).

Some of the polymastigote and most of the hypermastigote flagellates of many species of termites and of the roach *Cryptocercus punctulatus* (32) are of special interest in that they apparently must have cellulose as their chief carbon source and soon die out if their host is fed on other carbohydrates. The ability of these flagellates to digest cellulose and in some way to provide food for their insect hosts was proved by the early work of Cleveland (26). Later work has only served to confirm and extend his conclusions (170, 171, 100, 101).

3. *Other intestinal protozoa.* Although, following the original work of Boeck and Drbohlav (14), a number of species of parasitic amoebae have been cultured, little is known concerning their nutritional requirements. One reason for this lack of knowledge is that they have not yet been grown free from bacteria. Cleveland and Sanders (33) obtained bacteria-free abscesses of *Entamoeba histolytica.* When the amoebae

from such abscesses were placed in various sterile culture media only slight multiplication occurred and the cultures died out within 14 days. The amoebae grew well if supplied with living pure cultures of various bacteria, but dead bacteria supported no growth. The work of Dobell and Laidlaw (41) and many others after them has shown that in the ordinary contaminated cultures of *E. histolytica* the addition of starch enhances growth and enables encystation to occur.

4. *The sporozoa.* Nothing is known concerning the nutritional requirements of this vast class of protozoa, almost all of which are intracellular parasites and none of which have ever been cultured *in vitro.* The so-called "cultures" of malarial parasites, including those most recently obtained by Hewitt (87), represent at best a short survival with the production of one asexual generation. Christophers and Fulton (24), using concentrated suspensions of monkey red blood cells 90 per cent of which contained large parasites of *Plasmodium knowlesi,* found that glucose was consumed, although its addition produced only a small increase in oxygen uptake.

II. INSECTS. The food of insects presents an almost infinite variety, but it may well be that the fundamental nutritional requirements of the different species are very uniform, but are satisfied in different ways and from different sources. Uvarov (177) pointed out the many unsolved problems connected with insect nutrition. Considerable progress, some of it summarized by Wigglesworth (182) and by Hoskins and Craig (97), has been made since then toward the solution of certain of these problems. In this review, emphasis will be laid on the more recent work which is leading to a knowledge of actual chemical substances required for the various activities of insects. The extensive literature concerned with digestive enzymes will be omitted, except as it indicates some special nutritional requirement.

*A. Carbon and nitrogen sources.* The carbohydrates and related compounds which insects are able to utilize have been thoroughly studied for a few species. Adult honey bees lived well on solutions of glucose, levulose, sucrose, maltose, and trehalose (151). They could also utilize melezitose and possibly mannose, but not raffinose, lactose, galactose, dextrin, starch, inulin, rhamnose, xylose, arabinose, mannite, amygdalin, or salicin. Vogel (179) suggested that the failure of the bees to survive on certain sugars in Phillips' experiments may have been due to the non-ingestion of these sugars, perhaps because they were tasteless. She repeated Phillips' work, using in all the solutions enough $\frac{M}{4}$ sucrose to provide each bee with 0.006 ml. per day. This amount of

sucrose did not prolong life over that of bees given water only. Of the sugars which bees could taste, glucose, fructose, $\alpha$-methyl glucoside, maltose, trehalose, melezitose and raffinose could all be utilized. Of the tasteless sugars, arabinose, xylose, galactose, mannite, dulcite, sorbite, and cellobiose were utilized while rhamnose, mannose, sorbose, lactose, and melibiose were not. Bertholf (13) placed honey bee larvae in carbohydrate solutions and compared the length of life (a matter of hours) with that of controls in water. Sucrose prolonged life 7.5 times over the water controls; maltose, melezitose, dextrose, and honeys about 4 times; trehalose and dextrin 3 times; galactose and lactose 2 times; starch and glycogen not at all.

Adults of the fleshfly *Calliphora erythrocephala*, which live one to two months on sucrose solution as compared with two to three days on pure water, could use glucose, fructose, galactose, mannose, sucrose, maltose, trehalose, melibiose, lactose, raffinose, melezitose, xylose, $\alpha$-methyl-d-glucoside, starch, glycogen, mannitol and sorbitol, but could not use sorbose, cellobiose, arabinose, rhamnose, $\beta$-methyl-d-glucoside, helicin, arbutin, salicin, inulin, erythritol, dulcitol, or inositol (52). All the substances which were utilized were as effective as cane sugar except xylose, lactose, starch, and glycogen, on which the flies lived only one or two weeks.

Sorbitol gave significantly longer life of *Calliphora* adults than any other carbohydrate (202). Glycol, dihydroxy-acetone, alanine, glycine, butyric acid, isobutyl alcohol, and lactic, pyruvic, succinic, malic, or citric acids did not permit survival of the flies, but on glycerol they were able to survive 6 to 8 days (202). Adults of the apple maggot survived about a month when fed dextrose, levulose, sucrose, maltose, galactose or honey, but only a few days when fed lactose or starch (203).

The larvae of *Tenebrio molitor* did not grow in the absence of carbohydrate (204). When added at a concentration of 20% to a basal diet of protein, salts, and yeast extract, starch, mannitol or levulose produced rapid growth of the mealworm larvae; glucose, sucrose, xylose, lactose or glycogen produced slow growth; while arabinose, galactose and inulin produced no growth.

The utilization of cellulose as a chief carbohydrate source by certain insects is a subject of special interest, since relatively few animals are able to digest this polysaccharide. Cleveland (27, 30) showed that termites, which feed almost exclusively on wood, are actually unable to use cellulose directly and must have this substance digested for them by certain of their intestinal flagellates. The same situation exists in the

wood-feeding roach, *Cryptocercus punctulatus* (32). Following the work of Cleveland, a notion arose that all insects which are able to utilize cellulose as a food can do so only by the grace of microörganisms of one sort or another living in symbiosis with them. Recent work shows definitely, however, that some insects which feed on wood do not utilize the cellulose but actually subsist on sugars, starch, etc., in the wood, while others, which have no symbionts, themselves produce a cellulase (157, 130, 131).

Almost nothing is known as to which fats, if any, must be present in the diet of insects. Becker (12) found that mealworms (*Tenebrio molitor*) got along very well on meal with its starch removed but did not eat defatted meal. Some species, such as the beet leafhopper (54), can synthesize fats from carbohydrates, and such species may not require dietary fat. *Blatella germanica* can synthesize fat from protein (137). In general, the nature of the fat deposited in the fat body of insects depends, as in mammals, on the nature of the fat in the diet. Such is the case, for example, in the beetle *Pachymerus dactris* (36) and in the larvae of *Lucilia sericata* (197).

The apparent utilization of wax by the caterpillars of *Galleria mellonella* provides another example of an unusual type of nutrient material. Larvae fed on the impurities of comb wax, or on albumin, cereal, or sugar with albumin, failed to grow, but they did grow very well if any of these nitrogenous foods was supplemented with pure wax (139). Wax could be replaced by either of its two main components, cerine and myricine, but not by palmitic or stearic acid. A mixture of a nitrogenous food with water permitted good growth and metamorphosis. Metalnikov concluded that the wax normally served in place of water in the perfectly dry comb. Dickman (40) confirmed Metalnikov's finding that much of the wax passing through the gut of a *Galleria* larva was assimilated. Duspiva (44) showed that *Galleria* larvae utilized the fatty acids, esters, and alcohols of wax, but not the hydrocarbons.

The meager evidence concerning the nitrogen needs of insects indicates that they require a full complement of amino acids. Pure silk fibroin did not suffice for the nutrition of larvae of *Anthrenus muscorum* but the combination of fibroin with sericin did (1). Incomplete proteins were insufficient for cockroaches (124) and for fleshfly larvae (141). *Drosophila* larvae require complete proteins or hydrolysates of complete proteins (109). Mixtures of amino acids, including threonine, did not support growth, suggesting that the amino acid requirements of *Drosophila* may be more complex than those of rats. Alcoholic yeast extract was present in all the media as a source of growth factors.

The ability of a few types of insects to utilize keratin, a protein which is not attacked by proteinases under the usual conditions, has been recently elucidated (110, 45). Such insects (as clothes' moth larvae, *Mallophaga*), and only such insects, show in the gut a strong reducing condition which makes keratin susceptible to digestion by ordinary proteolytic enzymes (67).

*B. Requirements for growth and differentiation.* 1. *Orthoptera.* The findings of McCay (124, 128) and Melampy and Maynard (137) show that *Blatella germanica* requires, in addition to carbohydrate and a complete protein, heat stable materials present in alcoholic yeast extract. On a diet of casein, starch, cellulose, sugar, lard, and minerals the roaches could live as long as two months, but no growth whatever occurred until yeast or yeast extract was added. The alcoholic yeast extract could not be replaced by a rice polishings extract containing both vitamins $B_1$ and G. The roaches also required an ether-soluble factor present in wheat.

2. *Isoptera.* Cook and Scott (37) found, contrary to Cleveland's (28) earlier results with *Reticulitermes*, that the termite *Zootermopsis angusticollis* lost weight on a diet of mercerized cotton changed daily. If the cotton was not changed, the termites gained as much weight as on a natural diet of wood not changed. Various foods were incorporated in a 2 per cent agar gel, itself of no nutritional value. The food and containers were changed daily. The termites gained weight on a diet of salts, casein, sucrose, or glucose, cod liver oil, and rice polishings extract. The omission of any constituent of the diet resulted in loss in weight.

3. *Lepidoptera.* Larvae of the Mediterranean flour moth, *Ephestia kuehniella*, required a fat soluble substance present in whole wheat, egg yolk, and butter (155). The larvae grew very slowly on a highly milled flour and the moths which emerged weighed only 50 to 60 per cent as much as those reared in whole wheat flour. Supplementing the highly milled flour with olive oil, lard, butter, ether extract of egg yolk, casein, or a salt mixture did not improve growth, but the addition of 1.4 per cent solids from a 50 per cent alcoholic extract of bakers' yeast did.

The growth of clothes' moth larvae (*Tineola bisselliella*) is greatly accelerated by yeast (35). Whereas a lot of 100 larvae on raw wool alone gained only 3 per cent in weight after 4 weeks, another lot fed on raw wool with yeast gained 32 per cent. The larvae grew normally on a diet of vitamins A and B free casein plus vitamins A and B free lactalbumin plus Harris yeast extract (38). *Galleria mellonella* larvae grew well on pollen or on various complete food mixtures incorporated

in honey and glycerin. Wax was not required. Growth was best in mixtures containing yeast, but second generation larvae reared on yeast with sawdust only were unable to metamorphose successfully and died in the late pupal stage (84).

The larvae of *Galleria mellonella,* which grew well on a diet of 1 part autoclaved yeast and 2 parts wax, did not grow if the autoclaved yeast was thoroughly extracted with water and alcohol (205). Such a deficient diet could be made suitable for normal growth and development by supplementing it with 5 to 10 $\gamma$ of nicotinic acid per 100 grams of medium.

Other positive results obtained with the feeding of known growth promoting substances to lepidopterous larvae are those of Grandori, Provasoli, and Giorgi (69). Certain races of silkworms, when fed or injected with tyrosine or lactoflavin, or a mixture of these two substances, spun heavier cocoons than the untreated controls. The effect of tyrosine was especially noticeable in groups reared on greenhouse leaves in April.

4. *Coleoptera.* Sweetman and Palmer (166) showed that *Tribolium confusum* did not grow on commercial wheat embryo extracted with alcohol and ether, but did grow if the alcoholic fraction alone was returned to the residue. The beetles grew normally on a diet of casein, dextrin, and salts only if it was supplemented with an alcoholic extract of wheat germ. The *T. confusum* larvae require at least two accessory factors, one contained in rice polishings extract, the other in autoclaved yeast (164). If the latter was present at a level of 1 per cent in the diet, the length of the larval stage was, within certain limits, inversely proportional to the amount of rice polishings extract supplied. *Tribolium* also requires phosphorus but not vitamin D (142). The other common flour beetle *Tenebrio molitor* does not grow and metamorphose on purified wheat flour or polished rice unless these are supplemented with a small percentage of the grain husks, or with egg white (149).

Two thorough studies of the nutritional requirements of larvae of *Tribolium confusum* have recently appeared (206, 207). In one (206), the basal diet contained casein and had to be supplemented in order for growth to occur with thiamin, riboflavin, an alkali-treated yeast extract ($B_x$), ergosterol and a water and ether insoluble material (factor U) present in yeast, liver and egg yolk. The optimal amounts of the known growth factors in 1.5 grams of diet were 6 $\gamma$ of thiamin and of riboflavin, and 100 $\gamma$ of ergosterol. The fat body of larvae kept on a diet deficient in thiamin or $B_x$ consisted of small cells poor in fat. In

the other study (207), the basal diet contained egg white and had to be supplemented with thiamin, riboflavin, alkali-treated yeast extract ($B_x$), ergosterol and histidine. It is concluded that in this diet Factor U was supplied in the egg white, whereas in the other diet histidine was supplied by the casein. The factor $B_x$ could not be replaced by bios I, II, or III, or combinations of them. *Oryzaephilus surinamensis*, whether with or without symbionts, had the same nutritional requirements as *Tribolium*, except that it appeared not to require Factor U (206).

Payne (150) has shown the need of two species of coleopterous larvae, *Synchroa punctata* and *Dendroides canadensis*, for special pupation inducing substances in their food. She placed some grown larvae of each species in vials with sterilized oak bark, while others were placed in vials with unsterilized oak bark or with mycelium of *Armillaria nigra*, a fungus commonly found in dead and dying trees. All of the two latter groups pupated and emerged as adults within a few months. Of the larvae kept on sterilized bark, none pupated, although many of them survived as apparently healthy larvae for 6 years. Every half year during this time a few of the larvae of this group were removed and fed on unsterilized oak or rhizomes of the fungus *A. nigra*. Such larvae pupated within 5 days, a dramatic effect of the essential pupation-inducing substances which must be present in *A. nigra* and possibly other microörganisms.

Koch (105) found that the yeast-like symbionts of *Silodrepa panicea*, present in the cells of special pouches at the anterior end of the midgut, furnish their host with essential growth factors. When symbiont-free larvae were placed on a diet of condensed pea soup, on which normal symbiont-containing larvae grow very well, they failed to grow. A corresponding group of symbiont-free larvae fed the pea soup with 25 per cent of irradiated dried yeast grew normally. The symbionts evidently enable the host to live on a diet deficient in certain growth factors which are present in yeast. There are at least two heat stable factors concerned (106).

Larvae of the *Lyctus* powder-post beetle do not utilize the skeletal substance of wood (148). They required for normal development a substance which could be extracted from oak sapwood with water at a temperature of 60°C. Extraction of the wood with fat solvents did not alter its suitability as food. The beetles were reared on wood-free diets containing starch, sugar, and protein.

The hide beetle, *Dermestes volpinus*, grew almost as well on a diet of

casein, cystine, cholesterol, salts, and 10 per cent yeast as on a control fish meal diet (56). On a diet of yeast and salts only, the larvae grew well at first but rapidly died off at the third molt. With yeast, salts, and cholesterol, growth was even better than on the fish meal diet. *D. volpinus* is the third species of insect shown to require cholesterol (see *Diptera* below).

5. *Diptera*. Delcourt and Guyénot (39) early recognized the importance of a strictly controlled environment for the proper interpretation of experiments concerned with inheritance in *Drosophila*. They succeeded in rearing the fruit flies under aseptic conditions with autoclaved yeast suspension soaked up on cotton as the sole food. Guyénot (71) showed that under sterile conditions *Drosophila* larvae require, in addition to peptone and salts, lecithin (or some impurity in it) and an alcohol-soluble fraction of yeast autolysate. Bacot and Harden (7) essentially confirmed these results. On a diet of purified caseinogen, starch, salts, and cane sugar, both butter fat and yeast extract were necessary for growth to occur. Yeast extract could be replaced by wheat germ extract. Butter fat appeared to supply some fat soluble material other than vitamin A.

Two of the growth factors supplied by yeast extract have been shown to be vitamin $B_1$ and lactoflavin (95, 96). However, these two pure substances together could not replace yeast extract in a diet consisting of a peptic digest of casein, sucrose, the unsaponifiable fraction of arachis oil, salts, and yeast extract. The unsaponifiable fraction of fat could be replaced by pure cholesterol or ergosterol or by larger quantities of sitosterol, stigmasterol, or phytosterol, but not by calciferol or lumisterol.

Tatum (168) has shown that *Drosophila* larvae require under sterile conditions three fractions of yeast in addition to their carbohydrate, amino acid, cholesterol, and known vitamin requirements. In the presence of any one of the three factors no growth occurs. Factors II and III together enable growth and development to occur but at a slower rate than in the presence of all three factors. If factor I and either II or III are present larval growth is rapid but the insects die as pupae. Tatum has also confirmed the need of *Drosophila* larvae for riboflavin and has demonstrated that they require nicotinic acid, $10\gamma$ per 10 ml. of medium being the minimum amount which had to be added to a medium of 1 per cent alcohol-extracted dry yeast, 1.5 per cent agar, and 1 per cent sucrose in order to permit normal growth.

Beadle, Tatum and Clancy (11) and Khouvine, Ephrussi and Chevais

(103) have recently found that partial starvation of *Drosophila* larvae induces the formation of the so-called $v^+$ hormone (responsible for eye pigmentation) in vermilion larvae which ordinarily lack it. The $v^+$ hormone is also synthesized from tryptophane by an unidentified bacillus, which, when ingested by vermilion larvae, modifies the eye color of the resulting adults (169).

Housefly larvae, like *Drosophila* larvae, feed in nature on media rich in microörganisms. Glaser (60) succeeded in growing *Musca domestica* under sterile conditions through several generations by using as a medium for the larvae a mash made from heated whole minced swine liver coagulum, dried brewers' yeast and fine pine sawdust.

The fleshfly *Lucilia sericata* has been extensively studied from the nutritional standpoint. Wollman (187) grew the larvae under sterile conditions on sterilized sheep's brain. Michelbacher, Hoskins and Herms (141) used a sterile synthetic diet consisting of highly purified casein, yeast, salts, butter or cod liver oil, and cystine. Growth was as good as on contaminated meat or fish. If the cystine was omitted, growth was good but the pupae were irregular in shape. Hobson (91) reared *Lucilia* larvae on an autoclaved diet of liver and yeast incorporated in nutrient agar. A factor present in yeast was shown to be essential if the larvae were kept under sterile conditions. Yeast autolysate contained at least two heat stable factors and one heat labile one. The latter was shown to be vitamin $B_1$ (92). Hobson (93, 94), working with *Lucilia*, demonstrated for the first time the requirement of an organism for cholesterol in its food. The basal diet consisted of a 15 per cent meat peptone solution, thoroughly extracted with ether to remove the last traces of fat, and a small amount of aqueous yeast extract. Under these conditions cholesterol was the only fat soluble substance required for growth. Other sterols were less active than cholesterol.

The nutrition of mosquito larvae has also attracted considerable attention. These insects, in common with the other dipterous larvae already discussed, ingest under natural conditions large quantities of microörganisms of all kinds. Barber (9, 10) was able to rear them on pure cultures of bacteria and protozoa, but all attempts to rear them in the absence of living microörganisms proved fruitless. Hinman (89) obtained the development of small numbers of bacteria-free *Aëdes aegypti* mosquitoes in water rich in organic matter and sterilized by filtration through a Berkefeld N candle. He later (90) attempted unsuccessfully to rear *A. aegypti* in various autoclaved media, on

bacteria killed by heating at 60°C. or by means of formaldehyde, and in filtrates from bacterial cultures. Trager (172) found that *Aëdes aegypti* larvae could be readily grown under sterile conditions in a medium consisting of an autoclaved 0.5 per cent solution of Lilly liver extract no. 343 and suitable amounts of a washed yeast suspension killed by heating for one-half hour at 80 to 85°C. Casein with autoclaved yeast extract could replace whole killed yeast (173). The larvae required at least two sets of growth factors, one present in liver extract, the other in yeast and yeast extract. The former set of factors has been studied in some detail (175). It was found that the liver extract contained a number of substances essential for the growth of *A. aegypti* larvae, one of them being riboflavin (176). In the presence of all the other growth factors supplied in partially purified form, crystalline riboflavin permitted normal larval growth at concentrations as low as 0.0004 mgm. per ml. and had a definite effect at a concentration of 0.00008 mgm. per ml. Thiamin, or certain of its degradation products, was also required. Pantothenic acid and vitamin $B_6$ are other essential factors (208). In addition to these organic growth factors, *A. aegypti* larvae require calcium (174), as do the larvae of *Theobaldia incidens* (53).

Frost et al. (53) studied the general nutritional requirements of *Theobaldia incidens*. When they attempted to rear larvae of this mosquito under sterile conditions in a heavy suspension of autoclaved bakers' yeast in distilled water, only about 25 per cent of the insects reached the adult stage. They did not try Trager's liver extract-killed yeast medium but instead did the rest of their work under non-sterile conditions. Hence no sound conclusions can be drawn from their results.

6. *Siphonaptera*. Dried blood alone or yeast alone did not support growth of the larvae of the rat flea *Nosopsyllus fasciatus*, but blood plus yeast enabled almost all of the larvae to reach the adult stage within 26 to 38 days (at 23°C.) (162). Both the serum and the red cells were essential, but hemoglobin or ferrous ammonium sulfate could be substituted for the red cells. The larvae grew on non-autoclaved red cells plus autoclaved serum plus autoclaved yeast, but did not grow if all the constituents were autoclaved.

7. *Hymenoptera*. It is well known that food is the factor which determines whether a female bee larva is to develop into a worker or into a queen. Larvae which receive the specially prepared food, "royal jelly," only during the first 3 days and then get honey and undigested pollen, develop into workers, whereas larvae which receive royal jelly

throughout the larval stage develop into queens. The problem as to what substances in royal jelly are responsible for this effect seems not to have attracted the attention it deserves. Aeppler (2) noted that royal jelly is high in protein and cystine and contains some vitamin B. The claim of Hill and Burdett (88) that royal jelly contains vitamin E appears to have been adequately refuted (134, 51).

Heyl (209) observed that the injection of pyridine extracts of royal jelly into immature rats increased the activity of the Graafian follicles, a result which Melampy and Stanley (210) could not repeat when they used acetone-dried royal jelly. Townsend and Lucas state in a preliminary report (211) that the ether soluble fraction of royal jelly when fed to *Drosophila* exerted a great influence on the rate of reaching sexual maturity and the number of eggs laid.

In connection with the problem of the effect of food on caste determination the remarkable results of Goetsch (68) with the ant *Pheidole pallidula* are worthy of note. Young, just fertilized queens of this ant lay small eggs from which only small, short-lived workers can develop, regardless of their food. Their fate is determined either by their genotypic constitution or possibly by the small amount of storage food in the eggs from which they hatch. The later, larger eggs can give rise to normal workers or to soldiers, and Goetsch found that the type of food determined which caste developed. Larvae fed pieces of insects, meat, or coagulated egg white developed into soldiers, while those fed sugar or honey developed into workers. Ant larvae given fluid protein such as blood, meat juice, or raw egg white developed into workers, as on sugar solution. Apparently a highly concentrated protein diet is necessary for the development of soldiers. Under natural conditions in a colony only a relatively few larvae would happen to find near them fragments of insects or meat. Consequently, only a few soldiers are developed, whereas the majority of the larvae get liquid food and become workers.

*C. Requirements for maintenance and reproduction.* Numerous species of insects can live in the adult stage and reproduce without taking any food at all. Others require only water, others carbohydrates, while others can live on a carbohydrate diet but must have other nutrients in order to reproduce. Still others appear to require a complex diet for maintenance as well as reproduction. In general, the greater the degree of development of the sex organs at the time the insect becomes adult, the simpler are its nutritional requirements during the adult stage.

Among the Lepidoptera, most species possess in the adult stage no

proteolytic enzymes whatever and frequently no enzyme other than invertase (163). In the latter group, water rather than sugar may be the essential substance. For example, adults of the oriental fruit moth lived as long and laid as many eggs when fed water as when fed 10 per cent sucrose solution (167). This problem has been investigated in greater detail by Norris (145) working with moths of the genus *Ephestia*. *E. kuehniella* could live and reproduce perfectly without any water, while *E. cautella* and *E. elutella* females, even if kept in an atmosphere of high relative humidity, required water in order to attain maximum egg laying and longevity. The males of the two latter species lived longer with water than without it. Females of *E. kuehniella* and *E. cautella* lived longer on a diet of sucrose solution than on distilled water, but did not lay more eggs. The feeding of albumin solution to *E. cautella* females had the same effect as distilled water. Norris concluded that the moth fat body has two main reserve foods, one serving to develop the ovaries and the other to maintain life. The latter, but not the former, can be partially replaced or supplemented by a sugar diet. Fecundity is not increased by the prolongation of life after exhaustion of the former substance.

The adults of the common Diptera have more complex requirements for maintenance and especially for reproduction than do the Lepidoptera. Glaser (58) found that houseflies died in 1 to 2 days if given no food. The feeding of a protein diet such as bouillon, blood serum, or egg white increased the longevity but slightly. A diet of sucrose alone increased the longevity considerably, but very few eggs were laid. A diet of sucrose plus bouillon increased the longevity still more and a great many eggs were laid. Evans (50) obtained similar results with the fleshfly *Lucilia sericata*. Sugar was necessary for growth of the imaginal fat body and maintenance of life in both sexes, while meat was essential for the development of the ovaries. Dorman, Hale and Hoskins (42) similarly found that various species of fleshflies did not develop their ovaries on a diet of sugar and water. After as long as 6 weeks on this diet, the addition of fish meat resulted in egg production within 10 days, the normal preoviposition period. Casein with butter and yeast, soluble casein, blood albumin, and Lemco beef extract could not replace fish meat, and when these substances were added to a sugar and water diet no eggs were laid.

Adults of the apple maggot lived a month on a diet of sugar and water but laid very few eggs (203). When fed protein and water they lived only a few days, but when fed sugar, protein and water they lived

a month and laid numerous eggs. Of various proteinaceous substances tried, proteose-peptone and yeast gave the highest oviposition rates.

Some species of blood-sucking insects resemble *Lucilia* in that the females must have a protein-rich food in order for the ovaries to develop. Most female mosquitoes appear to require blood, but Huff (99) showed conclusively that some *Culex pipiens* were able to lay eggs without a blood meal and in one case without any food at all. At the same time Roubaud (159) and Boissezon (15) independently found that certain *Culex pipiens* females could lay a first raft of eggs without taking any food during the adult stage. These females have been found to belong to the so-called autogenous race of *C. pipiens* (160, 180, 132).

Among all species of blood-sucking mosquitoes other than autogenous *C. pipiens*, the females require a blood meal for egg formation and, in the case of hibernating forms, for fat body formation (86). The rôle of blood in ovulation has recently been studied for two species. Roy (161) observed that for the development by *Aëdes aegypti* of a first batch of eggs at least 0.82 mgm. of blood had to be ingested at one meal. Within limits, the number of eggs laid was approximately proportional to the size of the blood meal. According to Woke (186) washed red cells, serum, heparinized plasma, and hemoglobin solution from laked red cells, when fed separately, all supported normal egg production by *Aëdes aegypti.* If females of *Anopheles elutus* were given a blood meal 24 hours after emergence, only a few formed ripe eggs, while many reached Christophers' stage II of ovarian development (138). More produced ripe eggs after a second blood meal. In females fed raisins only, no eggs matured but the ovaries reached stage II. Such females, if given one blood meal after 7 days on the raisin diet, formed mature eggs in practically all instances. The feeding of washed red cells in saline induced maturation of the eggs of *A. elutus* (193). If the females were fed hemoglobin solution the ovaries reached stage II but no ripe eggs formed; if they were fed serum the ovaries did not even reach stage II. However, if insects previously fed raisins or sugar, and hence with their ovaries already in stage II, were fed serum the ovaries matured, whereas if they were given hemoglobin solution the ovaries did not mature. Hemoglobin solution, like sugar, raisins, etc., can bring the ovaries only to stage II. In order for the eggs to mature, another specific factor, present in serum and in the stroma of red blood cells, is required. The factor in serum resisted heating at 60°C. for 1 hour and was only partially inactivated by 30 minutes at 100°C. Heating serum at 100°C. for 15 minutes produced a coagulum which was found to

contain the activity. Ether extraction of serum did not remove the active substance. It is interesting to note that Glaser (58) had obtained similar results with the biting fly *Stomoxys calcitrans*. This insect could be fed on drops of defibrinated blood previously warmed to 35 to 37°C. On this diet the flies lived 20 days or more and laid many eggs. On serum alone or blood cells alone the longevity was lower and no eggs were laid. Recombination of the two blood fractions gave normal longevity and oviposition.

It is a remarkable fact that intracellular microörganisms or symbionts (18) occur in all insects and related arthropods which feed exclusively on blood throughout the life cycle. The Triatomidae, which were formerly considered to be an exception to this rule, have been shown (183) to contain organisms of a diphtheroid type which inhabit certain cells of the alimentary tract. Few of the insects which feed on blood during only a part of their life cycle contain such symbionts. Sterile blood is an insufficient diet for mosquito larvae and fleshfly larvae, whereas blood infected with bacteria is adequate. These facts led Wigglesworth (181) and Aschner (4) independently to suggest the hypothesis that the symbionts of the strictly blood-feeding forms furnish essential accessory growth substances. The only direct evidence for this is that of Aschner and Ries (6), and Aschner (5), who showed clearly that removal of the symbionts of the body louse, *Pediculus corporis*, resulted in cessation of growth and death in young lice and grave interference with reproduction in adult females. The experiments were so designed that it was evident that the procedures used to remove the symbionts were not in themselves deleterious. Some evidence was also obtained indicating that the deficiency produced by removal of the symbionts could be partly compensated by the intrarectal feeding of yeast extract.

Haydak (82, 83, 85) observed effects of food on the activities of adult worker honey bees (*Apis mellifica*). The nitrogen content of the young worker bees had to increase to a certain level before brood rearing began. When bees which had started brood rearing on a normal diet were confined to a diet of sugar and water, normal nursing activity continued for only one week. All the larvae fed after that period were unable to mature and were removed by the workers, the nitrogen content of which was considerably lower than at the beginning of the experiment. Bees in experimental colonies which were fed honey with pollen, meat scrap, or commercial casein developed their thoraces with respect to dry weight and nitrogen content at about the

same rate and to the same extent. On cottonseed meal, the dry weight increased normally but the nitrogen increase was slower. On dried blood, digested tankage, whole wheat flour, whole oats flour, corn flour, or pea flour the increase in dry weight and nitrogen was much below normal. On meat scrap and cottonseed meal the mortality was almost as low as on pollen, but it was much higher on the other foods. Brood rearing started on the three best foods of meat scrap, cottonseed meal, and casein, but was carried through only on the first of these diets. On all the other foods the larvae were removed from their cells at an early age. Removal of the queen from such colonies did not stimulate brood rearing. The degree of brood rearing activity on the different foods corresponded in general to their richness in protein, potassium, phosphorus, and sulfur.

III. OTHER INVERTEBRATES. So little is known concerning the nutritional requirements of invertebrates other than protozoa and insects that they can be conveniently considered in one section in a taxonomic order. Work concerned exclusively with digestive enzymes will not be reviewed.

*A. Coelenterata.* Hammett and his associates (77–81) have studied the effects on *Obelia geniculata* of feeding various pure substances known to be among the constituents of living cells. Sulfhydryl stimulated the proliferation of new hydranths, but did not affect differentiation. Glycine specifically accelerated regeneration; alanine had no effect; phenylalanine slightly favored differentiation; glutamic acid hastened differentiation and organization; tryptophane retarded catabolism; histidine similarly acted as an indirect stimulus to general growth by its sustaining effect on metabolic expenditure; d-arginine favored recurrent growth and retarded differentiation, regeneration, and metaplasia. Uracil, hypoxanthine and xanthine had the same effect as d-arginine; d-ribose had no effect; allantoin favored growth in general; adenine favored organization but retarded new growth formation.

*B. Plathelminthes.* The nutrition of *Planaria maculata* has been the subject of a number of interesting investigations. Wulzen (188) found that growth was less on heated mammalian tissue fragments than on unheated ones. The heat labile factor could be extracted with ether (189). The eosinophilic portion of beef pituitary had much more growth promoting power than the basophilic portion (190). If egg white was incorporated into an already adequate diet of liver pulp and starch it exerted a toxic effect when present in certain proportions (191). Wulzen and Bahrs (192) fed guinea pigs for one month on

adequate diets in which vitamin C was supplied to three different groups as: 1, orange juice; 2, tomato juice; 3, fresh grass or fresh kale. The fresh tissues of the guinea pigs were fed to planaria. On spleen from all groups of guinea pigs the worms remained healthy and grew rapidly. On lung they remained healthy and grew fairly well. On liver, good growth occurred only among the worms given liver from guinea pigs which had been fed kale. The next best growth was obtained on the livers of grass-fed guinea pigs. On the livers of guinea pigs fed orange juice, and more especially tomato juice, the planarians showed by the third week a deficiency disease consisting of decreased growth, roughening of edges, development of irregular lumps, darkening of color, and a twisting out of shape. Similar results were obtained with heart and kidney. The planarian disease did not develop on these organs taken from guinea pigs which were fed both kale and tomato juice, showing that there was no toxic effect of the juice. The guinea pigs on the different diets all appeared equally healthy. It may be noted, however, that grass does contain substances, other than the recognized vitamins, which are essential for the growth of guinea pigs (108). Bahrs and Wulzen (8) have also found that spleen, lung, liver, heart, or kidney, from guinea pigs kept on diets deficient in any of the known vitamins, had a lower growth promoting power for planaria than did tissues from normal animals. Greenberg and Schmidt (70) found in liver a heat labile, ether soluble factor which, if added to a lean beef diet, markedly increased the growth of *Planaria maculata*. The substance could not be replaced by liver phosphatide, cephalin, lecithin, sphingospongin, sterols, known vitamins, unsaturated fats, fatty acids, auxin, or aqueous liver extract. The factor was present in whole yeast. It resembled in many respects McCay's factor H for trout (126).

*C. Nemathelminthes.* The free-living nematodes can be readily cultured on various media if living microörganisms, especially bacteria, are present. Metcalf (140) sterilized the eggs of *Rhabditis brevispina* by washing in sterile water and plating out on agar. In a sterile asparagus juice agar, larvae which hatched from sterile eggs were able to grow only past the first molt. But sterile larvae, hatched out in a sterile filtrate from a *Fusarium* culture, developed rapidly and normally.

The vinegar eel, *Anguillula oxophila*, can develop under natural conditions in various fermenting media, and is known to feed on bacteria. Zimmerman (198) succeeded in freeing worms of this species from microörganisms by repeated treatments with peroxide and sterile water. Such sterile worms, if placed in sterile vinegar, lived 11 to 16

days but did not grow. They also did not grow on autoclaved flour paste. The worms did grow under sterile conditions in a medium prepared by allowing flour paste to putrefy, filtering it, and autoclaving the turbid filtrate. Sterilized worms placed in sterile 2 per cent peptone solution with 0.5 per cent sodium chloride, or in beef broth, lived for some time but never reached sexual maturity. The addition of lecithin gave no improvement. In a medium consisting of yeast autolysate and peptone the worms lived for months but did not reproduce. If lecithin was added to the medium, they soon began to multiply. A medium exactly like that used by Guyénot for *Drosophila* larvae (see section II, *Diptera*), consisting of peptone, salt, lecithin, and an alcoholic extract of yeast autolysate, gave rapid growth and multiplication of the worms.

Glaser (59) succeeded in culturing *Neoaplectana glaseri*, a nematode which is taxonomically closely related to the free-living forms, but is parasitic in Japanese beetle grubs. The nematode was grown on plates of dextrose veal infusion agar covered with a growth of living yeast. Chance-mixed bacteria were present. Under such conditions of culture, three to four generations developed in a period of two weeks before transfer to fresh medium was necessary. After about six such transfers (18 to 24 generations) the female worms apparently became sterile and the cultures consequently died out. They could be saved by infecting live beetle grubs with them. After several grub passages, the worms could again be cultured *in vitro* for 18 to 24 generations, when they would again die out. It was then found (212) that if the cultures, just before they began to die out, were supplied with a powder made from dried beetle grubs, or with powdered whole bovine ovarian substance, they revived. In the presence of the ovarian substance the worms have been kept in culture continuously for several years and apparently can be kept indefinitely. Various proteinaceous substances which were tried could not be substituted for ovarian powder or beetle powder. The active substance in the ovarian powder was destroyed by sterilization in the autoclave but not by Arnold steam sterilization. In the absence of the ovarian powder the cultures waned, apparently because of a gradually accumulated deficiency of a nutritional factor essential for reproduction.

Study of the nutritional requirements of this parasite of Japanese beetles will be facilitated by a method, recently developed, for their culture free from microörganisms (213).

The free-living stages of the parasitic nematodes of vertebrates, like

Hence both the similarities and differences between the nutritional requirements of vertebrates and invertebrates have a great significance. Organisms of neither group can synthesize thiamin or riboflavin. On the other hand, vertebrates can synthesize cholesterol but not vitamin D, whereas certain invertebrates cannot synthesize cholesterol. Whether these organisms can synthesize vitamin D is not known. Perhaps in them cholesterol is somehow concerned with calcium and phosphorus metabolism, as the related sterols of the vitamin D group are in vertebrates. A knowledge of the function of cholesterol in the growth of organisms which require it as a nutrient, a knowledge of the rôle of organic acids in the growth of certain flagellates, or of the nature of the nutrient substances which affect caste determination in the social Hymenoptera, in short, a more penetrating knowledge concerning the nutritional requirements of invertebrates cannot help but be important to a better understanding of the general physiology of organisms.

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The free-living stages of the parasitic nematodes of vertebrates,

the free-living nematodes, also feed on bacteria. McCoy (129) found that larvae of the dog hookworm, *Ancylostoma caninum*, developed to the infective stage in pure cultures of various species of bacteria and in saline suspensions of live bacteria, but not in the presence of killed bacteria only. Glaser and Stoll (65) were able to rear to the infective stage, under sterile conditions, the larvae of *Hemonchus contortus*, the sheep stomach worm. The medium consisted of a fragment of fresh sterile rabbit kidney, heat-killed ground yeast, and liver extract (Eli Lilly no. 343) all incorporated in a soft semi-solid agar gel.

In a somewhat similar but more complex medium, Glaser and Stoll (66) have also obtained partial development of the parasitic stages of *Hemonchus contortus*. Before this work was accomplished, no helminth parasite of a vertebrate host had ever been induced to grow anywhere outside of a living animal.

*D. Mollusca.* It is now generally admitted that diatoms and other minute marine forms, including small animals (144), are the chief food of oysters and related Lamellibranchs (143, 194, 195, 25). The nature of the substances which they must obtain from this food is quite unknown. A beginning toward a more detailed study of the nutritional requirements of oysters was made by Martin (133), who fed oysters on pure cultures of several species of plankton organisms and found that the oysters grew better on some than on others.

Howes and Whellock (98), in the only study of its kind conducted with a mollusc, found that the snail *Helix pomatia* did not grow on a diet containing casein, glucose, lactose, starch, dried yeast, cystine, calcium carbonate, a complete salt mixture, filter paper, and lemon juice unless this was supplemented with both cholesterol and leaf extract or cod liver oil. Since the cholesterol was not quite pure, it may have been supplying some other sterol which was actually the essential substance. There was little growth if the dried yeast was omitted from the otherwise complete diet.

*E. Arthropoda. Crustacea.* The Cladocera, which have proved useful in various types of experimental work, are ordinarily reared in media, such as manure infusion, which are rich in microörganisms. Stuart, McPherson and Cooper (165) freed adult female *Moina macrocopa* from contaminating microörganisms and studied their nutritional requirements. The Cladocera grew well in pure cultures of live bacteria. In sterile water the *Moina* died without producing young. In autoclaved pond water, in Berkefeld-filtered or autoclaved normal medium, and in broth, 30 to 40 per cent of the *Moina* produced young. If killed

*Bacillus coli* were added to any of these media 40 to 50 per cent of the *Moina* produced a first clutch of young. In no case were continuous growth and reproduction obtained under sterile conditions. Similar results were obtained by Gellis and Clarke (57) with *Daphnia magna*.

Viehoever and Cohen (178) found that the growth, ovarian function, and reproduction of young parthenogenetic females of *Daphnia magna* were greatly inhibited in a medium made from sheep manure which had been extracted with petroleum ether. If small amounts of Triticol (cold-pressed wheat germ oil) were added to the same medium, growth and reproduction were normal. The authors interpret these results as indicating a need of *Daphnia* for vitamin E. Since microörganisms were present, the effect may have been an indirect one via some effect on the microörganisms. Moreover, Triticol contains a number of substances, other than vitamin E, which might have been the effective factors.

IV. General discussion. Knight (104) classified bacteria in groups having decreasing synthetic abilities, a type of classification already suggested by A. Lwoff (113) for microörganisms in general. It is of interest to attempt to place the invertebrates in Knight's scheme. His first main group consists of the autotrophic bacteria which assimilate nitrogen from inorganic sources and carbon from carbon dioxide. They obtain energy for the reduction of carbon dioxide either from light or from the oxidation of simple inorganic compounds. With the exception of the still very doubtful case of *Chilomonas paramecium*, no invertebrate falls in this group. Bacteria of the second group obtain their energy and carbon for assimilation from carbon compounds already more reduced than carbon dioxide, which is not assimilated. They derive their nitrogen from simple inorganic compounds. In this group could be included only a few of the colorless plant-like flagellates. Bacteria of the third group differ from those of the second only in requiring their nitrogen already combined as an amino acid, while those of the fourth group require, like vertebrates, a whole array of amino acids and also accessory growth-promoting substances. It is apparent that, at least on the basis of our present knowledge, all of the invertebrates except the colorless plant-like flagellates fall in this fourth group. Even the simplest free-living ciliates require numerous amino acids and at least one growth factor (thiamin). The metazoan invertebrates which have been studied have nutritional requirements at least as complex as those of vertebrates or of the most highly parasitic bacteria and protozoa.

Hence both the similarities and differences between the nutritional requirements of vertebrates and invertebrates have a great significance. Organisms of neither group can synthesize thiamin or riboflavin. On the other hand, vertebrates can synthesize cholesterol but not vitamin D, whereas certain invertebrates cannot synthesize cholesterol. Whether these organisms can synthesize vitamin D is not known. Perhaps in them cholesterol is somehow concerned with calcium and phosphorus metabolism, as the related sterols of the vitamin D group are in vertebrates. A knowledge of the function of cholesterol in the growth of organisms which require it as a nutrient, a knowledge of the rôle of organic acids in the growth of certain flagellates, or of the nature of the nutrient substances which affect caste determination in the social Hymenoptera, in short, a more penetrating knowledge concerning the nutritional requirements of invertebrates cannot help but be important to a better understanding of the general physiology of organisms.

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# THE SECRETIONS OF THE INTESTINE

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For the purpose of this review the intestine is divided into three parts which have clear cut structural differences and which have distinctive functions to perform: *A*, the part of the duodenum just distal to the pylorus, containing the duodenal or Brunner's glands; *B*, the rest of the duodenum, the jejunum and the ileum; *C*, the cecum and colon.

The review is concerned only with mammals, and with the external secretions of the intestine.

A. THE BRUNNER'S GLAND AREA. *Anatomy.* The first part of the duodenum has embedded in its mucosa and submucosa the duodenal glands. These structures, which in many species appear to be continuous with the pyloric glands (for extensive discussion of this and other histological points see Villemin (1922), Plenk (1932), Patzelt (1936)), occupy both the mucosa and submucosa near the pylorus, but as they pass distally soon come to lie wholly beneath the muscularis mucosae. They are composed of ramifying tubules which empty by many ducts into the bases or sides of the crypts of Lieberkühn in the overlying mucosa. The cells which make up the alveoli of the glands are histologically of the mucous type, though in the rabbit and hare "serous" cells are also present (Tschassownikow, 1928a, b; Haase, 1937). Villemin (1922) states that they are also present in the horse.

*Characters of the juice.* Colin (1854), by his acute experiments on the horse, seems to have been the first to collect and accurately describe duodenal juice. Although extracts of the upper part of the duodenum were investigated by a certain number of subsequent workers, its secretion was not examined again till 1902, when Ponomarew (1902), working in Pavlov's laboratory, used fistula methods. He described the secretion from the first part of the duodenum of two dogs as a colorless syrup-like fluid, composed of a clear, fluid part and light gray mucus.

Recently the secretion of the upper part of the duodenum was extensively reinvestigated (Florey and Harding, 1933a, 1934, 1935a,

b; Wright *et al.*, 1940). The physical characters of the juice were usually those described by Ponomarew, though some of his conclusions on the control of the secretion were not substantiated. Utilizing both "acute" preparations and fistulae of various types, the secretions were collected from the cat, dog, goat, sheep, rabbit and pig. No differences were found between the juices from denervated and innervated fistulae (pig, cat, rabbit). From good clean fistulae the juices were clear, either colorless or faintly yellow. Deposit was often inappreciable even after centrifuging but when present consisted of grayish mucin (probably from the goblet cells of the villi) and cell débris, for the most part of unrecognizable origin though no doubt much of it epithelial. Bacteria were also found.

The specific gravity varied between 1007 and 1009. The juices from all the five species were mucoid, but the degree of stickiness varied from species to species. Juice from fistulae of the rabbit, goat and sheep was thicker and more like egg-white than that from the pig, cat and dog, which pulled out into long tenacious threads. Acetic acid precipitated the mucin in the first group but only produced a faint haze in the second. It was shown that both types of mucin produced large amounts of reducing substances when hydrolyzed, one of the accepted chemical characteristics of true mucins. In parenthesis, it may here be remarked that very little is known of the chemical composition and ultimate fate of secreted mucin in the gastro-intestinal tract; the subject might well repay careful investigation.

*Chemical composition.* Ponomarew (1902) noted that dog's duodenal juice was alkaline and estimated that the alkalinity was equal to that of 0.09 to 0.15 per cent $Na_2CO_3$. Florey and Harding (1933a, 1934) investigated the juice of several species and found that that of the goat had very little neutralizing power (1 cc. equivalent to 0.04 cc. N/10 soda), but that 1 cc. of rabbit fistula juice was equivalent to as much as 0.75 cc. N/10 soda. Rabbit juice obtained in acute experiments gave even higher figures (e.g., 1 cc. equivalent to 1.32 cc. N/10 soda). The values for cat, dog and pig juice lay between those for the goat and rabbit, 1 cc. being equivalent to from 0.25 to 0.5 cc. N/10 soda. All these juices had a very alkaline pH at the end of the collection period, no doubt partly due to the loss of $CO_2$ to the air during collection. Rabbit juice sometimes became as alkaline as pH 9.3 but when it was collected under oil values of pH 8.0 to 8.2 were obtained. The pH of goat juice was 8.2 to 8.4, pig juice 8.4 to 8.9, cat juice 8.7 to 8.9, dog juice 8.4 and sheep juice 8.3 to 8.4, when collected in contact with air. That the

buffering power of the juice was mainly dependent on bicarbonate and not on mucin was shown by Havard (1934).

The total solids of fistula juices varied from 1.5 per cent for the goat to 1.2 per cent for the pig, slightly more than half the solids being inorganic. No detailed examination of the mineral content was made but there appeared to be considerable amounts of sodium chloride present. Mucin, no doubt, makes up the greater part of the organic solids.

*Enzymes.* The duodenal enzymes have been investigated by making extracts of the mucosa and by examining the secreted juice. For determining what enzymes are actually secreted the former method is useless, although enzymes destined to be secreted into the lumen may, among others, be found in the mucosa. Ponomarew (1902) reported that the juice contained invertase, lipase and a feeble or inconstant amylase, as well as pepsin-like and rennin-like enzymes; the presence of the last two was confirmed by Pavlov and Parastschuk (1904) and of the pepsin-like enzyme by Abderhalden and Rona (1906). Florey and Harding (1934) reported the presence of a pepsin-like enzyme in goat and dog juice and a trace of lipase in the dog. Ponomarew found enterokinase.

A comparative investigation was recently made (Wright *et al.*, 1940) on the enzyme content of spontaneously secreted juice from a number of species and also on that of juice collected after vagal stimulation, sympathetic section and eserine administration in the cat. Care was taken to avoid bacterial contamination and to keep the cellular content to a minimum. In addition good chemical methods were used. The tests for proteases were consistently negative; no trace of a pepsin-like, trypsin-like or cathepsin-like enzyme was ever found. Tests for dipeptidase and polypeptidase were nearly always negative; significant splitting of these substrates ran parallel with exceptional cellularity of the juice. There was no evidence that cell-free juice contained significant amounts of invertase. Traces of lipase were occasionally found. The importance of the cellular content was indicated by the fact that addition of intestinal villi to juice which was negative for dipeptidase and invertase gave it the property of splitting dipeptides and cane-sugar.

Whatever their method of production, the juices always contained amylase. With a very few unexplained exceptions they were also capable of fully activating pancreatic juice to digest protein at an alkaline pH (enterokinase). In this connection it is noteworthy that Kunitz

(1938–39) used the secretion present in the duodenums of pigs after slaughter as a source of material for purifying enterokinase.

A bacteriolytic enzyme, lysozyme, discovered by Fleming (1922) was also found in appreciable quantities in pig juice, but only in traces in the other species tested.

CONTROL OF SECRETION. It should be emphasized that in all species the typical mucoid juice could be collected without subjecting the fistula to mechanical stimulation, e.g., by a catheter. This sharply differentiates fistulae of the upper duodenum from those of other parts of the intestine, which, except for small amounts of "periodic" juice (Boldyreff, 1904, 1928), will only produce fluid after some form of local stimulation.

*Effect of local stimulation.* Ponomarew (1902) showed that the introduction into the fistula of undiluted gastric juice, 0.25 per cent HCl, the products of the digestion of fibrin by gastric juice, one drop of mustard oil in 200 cc. water, and even normal saline increased the flow of juice. Fatty substances gave little or no increase. His work was extended by Florey and Harding (1933 a, 1934) who investigated the effects of perfusing dilute HCl (usually N/10 or less) through the first part of the duodenum and found that the fluid increased in volume during its passage and became mucoid. A considerable degree of neutralization also occurred. Histological evidence was obtained from the cat, rabbit and guinea pig that the passage of HCl exhausted the mucin-containing cells of the duodenal glands. The neutralization and the production of mucus were not inhibited by atropine. The perfusion of N/20 $H_2SO_4$ also caused secretion, but when dilute mustard oil (one drop in 100–150 cc. water) was perfused fluid was only produced concurrently with the histological picture of inflammation.

*The influence of food.* Ponomarew (1902) reported that feeding did not influence the flow of juice from duodenal fistulae in the dog. Cats, dogs and pigs (Florey and Harding, 1934, 1935a, b; Wright *et al.*, 1940) have lately been investigated. When starved for 24 hours the fistulae ceased to secrete or only produced a few drops an hour; within a few minutes of taking food juice began to flow. A similar phenomenon was observed in a goat. Later the same was shown in cats and pigs, in duodenums which had been transplanted subcutaneously and deprived of all nervous and vascular connections with the abdominal contents. A similar denervated fistula in the rabbit secreted juice continuously, probably because the stomach of the rabbit is never empty. Clearly, in the cat and pig at least, some form of hormonal

stimulus was at work. Highly purified secretin injected intravenously in the cat caused typical secretion from a quiescent fistula. Cruder secretin preparations were active on innervated fistulae in the dog.

Fogelson and Bachrach (1939), working with dogs, confirmed the finding that upper duodenal fistulae secreted a mucoid juice, the volume of which was increased by a mixed meal. They too found that secretin given intravenously produced a flow of mucoid duodenal juice; they showed that the flow was accompanied by muscular contractions and their conclusion was that no proof had yet been offered of such secretion being independent of muscular action.

Ponomarew (1902) reported that 0.5 per cent HCl inserted into the stomach increased the secretion from a dog fistula slightly, while olive oil and 5 per cent and 10 per cent solutions of sodium oleinate increased the secretion markedly. When these experiments were repeated on cats (Florey and Harding, 1935b), olive oil and cane sugar did not cause secretion but meat and milk invariably caused a flow of juice within 3 to 4 minutes of ingestion. The insertion of N/10 or N/5 HCl into the stomach also produced juice but the amount was never as much as after meat and milk.

*The influence of nerves.* It was recently shown (Wright *et al.*, 1940) that prolonged stimulation of the infracardiac vagi of decerebrate or decapitate cats regularly produced a considerable flow of mucoid juice from the first part of the duodenum. This juice was indistinguishable in its physical characters from that collected from a permanent fistula, whether innervated or denervated, and its production was accompanied by histologically demonstrable exhaustion of the mucus-containing cells of Brunner's glands. This outspoken effect of vagal stimulation was abolished by certain of the barbiturate anesthetics and all anesthetics tested had some inhibitory effect.

In addition to the vagal effect, it was found that cutting the greater splanchnic nerves in the thorax was followed by secretion of typical mucoid juice from the first part of the duodenum. Large doses of eserine subcutaneously gave a similar secretion; if acetyl choline was given to an eserinized cat pieces of mucosa were often shed into the juice.

It appears from these results that the part of the duodenum containing Brunner's glands is controlled by a hormone, possibly secretin, and has in addition a sensitive acetyl-choline mechanism which can be set in motion by vagal stimulation, by the removal of a sympathetic nerve inhibition or by paralyzing the esterase with eserine. The fact that

pilocarpine produces juice from the duodenum is supporting evidence for the participation of the vagus in the control of the duodenal secretion.

It has been stated that the administration of histamine causes a flow of intestinal juice. In the first part of the duodenum this drug appeared to be without effect.

*The amount of juice secreted.* Ponomarew (1902) stated that his two dogs produced 0.23 to 1.3 cc. an hour and 0.06 to 1.08 cc. an hour respectively. Florey and Harding (1934) obtained a steady 1.5 cc. an hour from a dog after food while Wright *et al.* (1940) obtained 1.5 cc. and 2.0 cc. an hour respectively from two fully innervated fistulae in dogs. Fogelson and Bachrach (1939) got as much as 8.8 cc. in an hour from a fed dog. From cat fistulae 0.25 to 0.5 cc. an hour was obtained. Relatively much larger quantities of juice are secreted by pigs, goats and rabbits, which have a greater development of Brunner's glands. From one pig as much as 17.0 cc. an hour was obtained and this from a fistula which did not by any means contain all the Brunner's glands. One large goat secreted 150 cc. in 7 hours, though most goats secreted considerably less than this. In rabbits, too, there was a relatively large production of duodenal juice though no fistula included more than a small part of the Brunner's glands. It might be thought that the continuous loss of some or all of the duodenal secretion from these fistulae would influence the general health of the animal, but no interference with growth or health was observed.

The quantity of juice produced from the duodenum in experiments on decerebrate or decapitate cats was considerably greater than that obtained from cat fistulae. A longer piece of duodenum was used in most of the acute experiments but there is good evidence that the juice came mainly from the upper 2 cm. (i.e., the Brunner's gland area). The quantities of clear sticky duodenal juice obtained from these acute preparations varied from 2 to 8 cc. per hour. Possibly a fistula never receives maximal nervous stimulation and therefore never secretes to full capacity. There is good evidence that a satisfactory fistula has a histologically normal mucosa, at least for several weeks, and an adequate blood supply. Volta (1920) reported on two Thiry fistulae of the intestine which he considered were functionally and anatomically normal after five years.

*The duodenum and hemopoiesis.* Meulengracht (1935), basing his work on Castle's (Castle and Minot, 1936) and Sturgis and Isaac's (1930) elucidation of the gastric factors involved in pernicious anemia, showed that extracts of that part of the duodenum containing Brunner's

glands were active in supplying "intrinsic factor" for the treatment of pernicious anemia. He suggested that the cardiac part of the stomach, the pyloric antrum and the first part of the duodenum, which have certain histological similarities, formed a "gland organ" which secreted the factor. Thompson (1937) also found that duodenal extracts were effective, though he did not say whether he thought Brunner's glands were responsible for the activity.

No one has yet shown whether juice secreted by the pig's duodenum (probably mainly from Brunner's glands) contains the "intrinsic factor." Goodfriend et al. (1938) obtained a polypeptide preparation from the duodenal juice of pigs and goats which gave a reticulocyte response in the guinea pig (Jacobson's test). Their preparation was active in extremely small doses (e.g., 0.04 $\mu$g) and gave a sufficiently appreciable and constant response for assay purposes. Their material was, however, inactive in clinical cases of pernicious anemia and its function in hemopoiesis, if any, has not been elucidated.

*Production of secretin.* Volborth (1925) showed secretin to be present in the débris of intestinal juice, but it is unlikely to be secreted into the lumen of the intestine and then reabsorbed. The fact that secretin is only active when administered intravenously is against the view that it is secreted with the duodenal juice.

*Functions of duodenal juice.* As the result of the investigations of Ponomarew (1902) and Dobromyslow (1903) it was suggested that one of the functions of duodenal juice was to assist in the digestion of fats by providing a proteolytic enzyme, active in acid juice, which would help to remove the connective tissue covering from fat-containing cells. It is difficult to see how such a ferment could act in the first part of the duodenum, especially as bile was said to inhibit the proteolysis in certain circumstances, and later investigations have failed to substantiate the presence of a proteolytic enzyme. As far as is at present known the only enzymes constantly present are amylase and enterokinase. The amylase is probably weak compared with pancreatic amylase (though no exact figures are available) and therefore not of much significance. The enterokinase, on the other hand, appears to be powerful, and it is no doubt of the greatest importance in activating the pancreatic juice as it emerges into the intestine. Whether duodenal juice contains more enterokinase than juice from the rest of the small intestine, as Pavlov believed, has not yet been determined.

It has long been thought that mucous secretions, by virtue of the physico-chemical properties of mucins, protect the underlying mucosa.

The alkalinity and high mucin content of duodenal juice suggest that the secretion of Brunner's glands may protect the delicate villi of the first part of the intestine from the damaging action of the ejected acid stomach contents. The possibility is supported by experiments on pigs (Florey *et al.*, 1939) in which it was shown that duodenum containing Brunner's glands was more resistant to gastric juice than other parts of the small intestine.

Villemin (1922), after studying the comparative anatomy of Brunner's glands, pointed out that with few exceptions they ceased at the entrance of the pancreatic duct but were independent of the entry of the bile duct. He inclined to the view that they took part in the neutralization of gastric juice.

This mucoid duodenal juice may also be useful in suspending food particles during intestinal digestion. Such a function appears particularly likely in the rabbit, where the chyme issuing from the pylorus is considerably diluted by the duodenal secretion (Florey and Harding, 1933b).

The possible function of the juice in hemopoiesis has already been mentioned.

Regarding the special contribution of Brunner's glands to the duodenal juice, it may be said that while the Brunner's gland area gives a good secretion in response to vagal stimulation, the adjacent few centimeters of the duodenum give a scanty secretion, and the rest of the small intestine none (in the cat; see section B). Similarly, in response to feeding, only fistulae which include the Brunner's gland area secrete spontaneously. Brunner's glands may therefore reasonably be held responsible for a large part of such secretions. There are good grounds for believing that the mucin is their product. It is impossible, however, to say precisely what are the contributions of the Brunner's glands on the one hand, and of the villi and crypts of Lieberkühn on the other, to the normal duodenal juice.

B. Small intestine. *Anatomy.* The mucosa lining the small intestine consists of tubular glands—the crypts of Lieberkühn—and projecting villi. Both are invested by a single layer of epithelial cells with other cells interspersed between. The investing layer consists of simple cuboidal or cylindrical cells and goblet cells, the latter reaching their fullest development on the villi; judging by the position of the many mitoses these cells all originate in the lower two-thirds of the crypts. Argentaffine cells scattered between the epithelial cells are found mainly in the upper part of the intestine; Paneth cells—cells with

large acidophil granules—are present at the bases of the crypts in some species, while the many small cells which lie between the epithelial cells are almost certainly lymphocytes. (The histological picture is discussed at length by V. Patzelt, 1936, p. 74, and the reader is referred to his article.) Our knowledge of the histology of the mucosa, which is considerable, contrasts with our lack of knowledge of the function of its several component cells. It has not, for instance, been settled whether the crypts secrete and the villi absorb or whether they have a common function, as the common origin of their epithelial covering might be taken to indicate, in which case both crypts and villi would secrete and absorb. Still less is anything certain known about the contribution, if any, of the Paneth and argentaffine cells to the secretion. The most that can be said about the functions of the lymphocytes is that some workers see in them a defence against harmful substances in the intestine (Hellman, 1934), though it has recently been shown that all the Peyer's patches can be removed from a rabbit or a rat without detriment to the health of the animal and without the formation of compensatory nodules (Sanders and Florey, 1940).

*Experimental methods.* Nearly all experiments have been done on the dog, and it is to be regretted that more is not known about the intestinal functions of other species. Two kinds of experiments have mainly been used—acute preparations, in which a loop of intestine is examined for a short time, and "chronic" or permanent fistulae, where the lumen of a loop of intestine is isolated from that of the main gut and made to empty externally.

Frerichs (1846) claimed that he was the first to use the acute preparation, and some of the latest workers have used it again. Chronic preparations have changed little, except for the general improvement in operative methods, since Thiry (1864) described and Vella (1888a) modified the fistula operation; preparations almost identical with theirs are generally used at the present time, though Johnston has described a modification which seems to have some advantages. Several kinds of fistulae, some not much used now, were devised in Pavlov's laboratory. Details of preparing the fistulae may be found in Markowitz (1937), Johnston (1932–33) and Babkin (1928, p. 760–762), who also gives further references.

Pavlov claimed to have made a fistula which was fully innervated. The interference needed to close the lumen, however, inevitably interrupts the submucous plexus. In all fistulae of the Thiry and Thiry-Vella types the muscle is cut, thus interrupting the myenteric plexus

as well; such fistulae are possibly deprived of at least part of their vagal supply but, at any rate while the pedicle remains healthy, retain their sympathetic supply.

Secretion by a fistula. *Spontaneous secretion.* It is an almost constant finding that fistulae of the jejunum or ileum do not secrete spontaneously in response to feeding. Boldyreff (1904), however, described a "periodic" spontaneous secretion, occurring at intervals of from 1 to 2 hours in starving dogs and inhibited by food. A fistula 20 to 30 cm. long would secrete up to 2 cc. of juice in 20 to 30 minutes and therefore, Boldyreff calculated, the whole intestine secreted about 30 cc. of juice at each period. Leper (1904), Brynk (1911) and Komarow (1924a) corroborated Boldyreff and the last noticed that atropine inhibited the secretion. Babkin and Ishikawa (1912) believed that the periodic secretion was related to increased gut movement. Boldyreff (1928) recently reaffirmed his opinion that periodic juice was a true intestinal secretion.

*Response to local stimulation.* In his original paper Thiry (1864) described how mechanical stimulation of an intestinal fistula by a sponge or catheter produced a flow of fluid, and at the present time an ordinary catheter or some modification such as Johnston's, is commonly used for obtaining fistula juice. Physiologists seem to accept such mechanically produced juice as true *succus entericus* though, as Boldyreff (1928) pointed out, it often contains blood. He considered it a pathological transudate, but he was not very consistent, as there is little or no material difference in his figures for the enzyme content of spontaneous periodic secretion and of juice produced by "gentle" stimulation. Pavlov (1910) regarded the *succus entericus* as a fluid whose composition was determined by the stimulus to secretion. Thus a mechanical stimulus excited a secretion of water to wash away the foreign body, and specific stimuli promoted the secretion of enzymes, notably pancreatic juice the production of enterokinase. This last was re-affirmed by Savich (1921). Similarly, Lombroso (1908) thought that fat and bile-salts, and Jansen (1910) that bile-salts alone, were the stimulus for lipase, but Orbéli (1917) could not confirm Jansen, and London and Dobrowolskaja (1910) denied that this kind of adaptation of enzyme to stimulus occurred.

Many chemical irritants have been found effective, e.g., acetic acid, 0.1 per cent HCl, 20 per cent $MgSO_4$, 0.25 per cent butyric acid, gastric secretion, mustard oil emulsion, triolein, gliadin albumose, lactose, erythro-dextrin, sodium oleate, ether, chloral hydrate, calomel (Babkin,

1928, p. 772). All of these substances except gastric juice have been used in much higher concentrations than are likely to occur normally.

Electrical stimulation apparently also causes some secretion. Dobroslawin (1870) described stimulating for ¼ hour periods with an electrode inside the fistula and collecting from 2 to 3 times as much juice during stimulation as during rest. A few other similar reports have been given (Thiry, 1864; Masloff, 1882; Bastianelli, 1892).

*Characters of fistula juice.* There is general agreement that the juice from a fistula consists of two parts—a watery supernatant fluid and a clumpy or floccular part. The fluid is slightly yellow, is clear or opalescent, has an aromatic smell and an alkaline reaction. The floccules are mainly composed of mucus with which are mixed degenerating epithelial cells, microörganisms and cholesterol crystals.

*Amount of juice collected.* The amounts which can be collected from a fistula by mechanical stimulation vary widely, even in one animal. De Beer *et al.* (1935) obtained from 64 to 175 cc. from a jejunal fistula in one dog, and from 10 to 60 cc. in another, in two hour collection periods; an ileal fistula yielded about 65 cc. in the first two hours of collection. Mosenthal (1911) holds the record with 115 cc. in one hour from an ileal fistula between 25 and 50 cm. long. Many workers have never obtained more than a few cubic centimeters (e.g., Andrejew and Georgiewsky, 1932; Nasset *et al.* 1935).

*Effect of denervation.* Molnár (1909) and Savich (1921) reported that denervated fistulae secreted more juice, especially in response to feeding, than fistulae with their nervous connections intact.

Nasset and his co-workers investigated the output of transplanted fistulae before and after cutting the pedicle (Pierce, Nasset and Murlin, 1935; Schiffrin and Nasset, 1939). In the latter paper they gave figures showing a decrease in the volume of jejunal secretion, both fed and fasting, after cutting the pedicle, but no change in the ileal secretion. They spoke also of differences in enzyme content, but as they estimated the enzymes of the whole juice, including the deposit, such extraneous sources as cells cast off from the mucosa were not excluded. They stated that the pH and the chloride and $CO_2$ content were unaffected.

It is certain that when the pedicle is allowed to atrophy gradually no noticeable change in the volume or character of the secretion takes place. Presumably the secretory mechanism, like the circulation, has time to become adjusted to new conditions.

*Chemical composition of fistula juice.* The amount of mucin is very

variable, depending on the amount of floccular material in the juice, and may be so low that only a slight turbidity develops with 2 per cent acetic acid.

The pH is usually given as 8 to 9 (e.g., Bickel and Kanitz (man), 1934; Amberg and Sawyer (dog), 1926) but de Beer *et al.* (1935), with more careful collection and better chemical methods, gave values of 6.30 to 7.28 for the jejunum and 7.61 for the ileum. Schiffrin and Nasset (1939) gave 6.9 to 8.6 for jejunum and ileum. The neutralizing value is variously given as from 0.02 to 0.67 per cent $Na_2CO_3$.

De Beer *et al.* give extensive tables of their analyses of jejunal, ileal and colonic fistula juice. The outstanding feature of their figures is the relative constancy of the individual kationic concentrations from one secretion to the other, and the constancy of the anionic sum with reciprocal variation of the Cl and $HCO_3$. Other experiments by the same workers show that an alteration of Cl content of the blood may be reflected in the *succus entericus* but not an increase in blood $HCO_3$.

For the various non-protein nitrogen fractions see Ikeda (1934), and for separate analyses of fluid and floccular parts of the juice, Amberg and Sawyer (1926).

*Nitrogen excretion.* The amount of nitrogenous matter secreted in the *succus entericus* was investigated by Mosenthal (1911). By making fistulae of a known proportion of the intestine in six dogs and collecting the secretion while following the total nitrogen intake and output, he deduced that the intestinal secretion might contain up to 35 per cent of the ingested nitrogen. Since the fecal nitrogen was usually only 10 per cent of the ingested nitrogen Mosenthal argued that at least 60 per cent of the nitrogen secreted into the lumen was reabsorbed. Hermann's (1890) closed loop experiment in which, over a period of weeks, mucin, desquamated epithelial cells and fat droplets formed a semi-solid mass, does not prove that in normal circumstances all this material is excreted with the feces. In such a preparation the material is not subjected to the digestive action of pancreatic juice and the physical action of bile, which would probably cause the death and digestion of almost the whole of this desquamated material.

*Lipid excretion.* Sperry (1926, 1926–27), and Sperry and Bloor (1924) have brought forward evidence that lipids are secreted by the intestine and, in normal dogs, largely reabsorbed, but escape with the feces when bile is excluded. Shapiro *et al.* (1936) by employing deuterium-containing fats, showed that 65 to 70 per cent of the diet fatty acids were absorbed in the absence of bile and that the increase of

fecal fat in the absence of bile was due to the fats secreted into the intestinal lumen.

ENZYMES. Much work on intestinal enzymes was done in the 19th century but, along with some of the more recent work, it left much to be desired for one or more of the following reasons; in particular quantitative comparisons should be treated with great reserve.

1. Chemical methods were primitive and often inadequate; buffers to keep the pH constant were, for instance, not known.

2. No precautions, or inadequate precautions, were taken to prevent bacterial enzymes from acting on the substrate.

3. No distinction was drawn between the properties of the juice which flows from a fistula, which, especially when collected by mechanical stimulation, contains cells, cell débris, and bacteria, and those of the pure secretion of the intestinal epithelium.

It has been abundantly shown that the juice from an intestinal fistula digests starch, cane-sugar, maltose, lactose, poly- and di-peptides and, to a small degree, fat. It also activates trypsinogen (enterokinase). But some of these enzymes are well known to be present in the cells of the intestinal mucosa, and even long centrifuging of the juice cannot eliminate enzymes which have escaped from ruptured cells. Amongst others Cajori (1933), Koskowski (1926) and Pierce *et al.* (1935) showed that whole juice digested more strongly than centrifuged juice, and the last two showed that digestion was still stronger if the juice was macerated with glass beads, which may be supposed to break up the cells in the deposit and liberate their enzymes.

The following paragraphs briefly survey some of the work on intestinal enzymes. Oppenheimer's *Die Fermente* (1925, 1926, 1936) is monumental and should be consulted.

*Peptidases.*[1] In the intestinal juice of dogs Salaskin (1902), Kutscher and Seemann (1902), Waldschmidt-Leitz and Waldschmidt-Gräser (1927) Cajori (1933) and others described a peptide splitting action. In man, Hamburger and Hekma (1902), Bickel and Kanitz (1934) and Owles (1937–38) found a similar action. Owles, and Pierce *et al.* (1935) preferred not to centrifuge the juice because this reduced the peptidase content. Cajori described a residual activity after centrifuging but found that the peptone was absorbed disproportionately quickly

[1] "Erepsin" was Cohnheim's name for the peptide-splitting ferment of the intestinal mucosa which he discovered in 1901. Several enzymes with distinctive properties, acting on peptides of different structure or complexity, are now separated, so the name becomes obsolete.

to the rate at which it was split by centrifuged juice. The common observation that after several hours of mechanical stimulation the peptidase activity of the secreted juice is reduced is usually explained as due to washing out of the enzyme from the mucosa, but may be due to removal of the desquamated cells from the lumen.

Waldschmidt-Leitz and Waldschmidt-Gräser (1927) and Glaessner and Stauber (1910), using dogs and rabbits respectively, found peptidase in the mucous membrane of gut to which the pancreatic juice had no access. The latter workers found none in the mucosa of a rabbit with diabetes due to destruction of the pancreas, and Foà (1908) found none in the mucosa of a six months old Vella fistula in a dog. Its absence from intestinal secretion could therefore on occasion be due not only to absence of cells but to the absence of peptidase from the cells. Linderstrøm-Lang (1939) after reviewing recent work considered the peptidases to be typical endo-enzymes.

*Enterokinase.* This enzyme was discovered in the *succus entericus* of dogs by Schepowalnikow (1899), working in Pavlov's laboratory, and in human juice by Hamburger and Hekma (1902), and the relatively few workers who have looked for it since have always found it present, (e.g., Falloise, 1904–05 (dog); Hermann and Ribère, 1931 (man)). It is developed already in early infancy, according to Austin (1909). Schepowalnikow found that the concentration in the mucosa was greatest in the duodenum and decreased downwards, and Pavlov believed that this was true also of the concentration in the juice. In the mucosa it is chiefly in the villi (Falloise, 1905). Ohno (1930) and Waldschmidt-Leitz and Waldschmidt-Gräser (1927) suggested that the lymphocytes which entered the gut might play a part in activating trypsinogen.

Sawitsch (1904) stated that in a loop of intestine deprived of pancreatic juice the secretion of enterokinase gradually failed. Unfortunately the paper is not available for consultation but the report given by Babkin (1928, p. 782) suggests that neither the methods used nor the results obtained justify this conclusion. The same criticism applies to the increase after food which he reported in 1917 (Orbéli and Sawich). Waldschmidt-Leitz and Harteneck (1925) suggested that pancreatic juice was necessary for the liberation of enterokinase from the epithelial cells but the evidence is indirect and not very convincing. Boldyreff (1928) regularly found it in dog fistula juice, and le Breton and Mocoroa (1931) specifically stated that it was present in the juice from a fistula several weeks old. Foà (1908), however, said that at 6 months it had gone from the juice. According to Savich (1921)

the falling off in enzyme concentration occurred only if the sympathetic nerve supply to the gut was intact, not otherwise.

Rasenkow's (1929) suggestion that enterokinase and secretin are identical cannot be considered seriously; his extracts may have contained almost anything. Comparative studies of the enterokinase in cell-free and cellular juice have not been made.

*Lipase.* Boldyreff (1904) was the first definitely to demonstrate an action of the *succus entericus* in splitting monobutyrin and fat, but recognized that the lipolysis was weak. Bickel and Kanitz (1934) found no lipase in juice from a human ileal fistula and Owles (1937–38) found very little in juice from the jejunal region obtained in man by the Miller-Abbott tube. Roger and Binet (1921) detected lipolytic activity only after the addition of bile to the juice. Reale (1932–1934) was convinced that lipase was not present in solution in fresh juice but that the concentration gradually rose with the progressive autolysis of desquamated cells. The finding of Roger and Binet and of Jansen (1910) and others, that bile increased the lipolytic acitivity of *succus entericus,* may therefore be due to destruction of cells by the bile.

*Amylase.* Dobroslawin (1870) first reported an amylolytic action in *succus entericus,* and since that time it has been found by almost every worker who has looked for it (see Babkin, 1928, p. 768; and Oppenheimer, 1925, Bd. I, p. 724). Bierry and Frouin (1906) found that removal of the cells by centrifuge and filter removed the amylase, but Cajori (1933) that it was less reduced than other enzymes by centrifuging. Owles (1937–38) found in temporarily isolated loops of human intestine that even if he did not remove the cells from the secretion the starch splitting activity rapidly diminished, apparently because of the washing out of pancreatic amylase. Wohlgemuth's (1910) observation that ligation of both ducts of the pancreas led to a disappearance of amylase from the feces also suggests that the intestine secretes an insignificant amount. Georgiewsky and Andrejew's (1934–35) observation on fistula bearing animals that a high starch diet increased the amylase in fistula juice is difficult to assess. Cajori found that starch was split by the juice and absorbed from the fistula at about the same rate; he found also that the amylase content of the juice was of the same order as that of the blood and other body fluids and suggested that the enzyme in the juice came from the blood.

*Invertase.* Leube reported an inverting action of *succus entericus* in 1868. Claude Bernard (1873) found activity in both the mucosa and the secretion. Euler and Svanberg (1921) found that the concen-

tration was greatest in the mucosa of the jejunum, less in the duodenum and least in the ileum. Tubby and Manning (1892) found invertase more often in the mucous than in the fluid part of the juice from a human ileal fistula; Bierry (1912) showed that when juice was centrifuged and passed through a Berkefeld filter it lost its inverting power, Koskowski (1926) that clear juice contained less invertase than turbid or cellular juice. Cajori (1933) and Röhmann and Nagano (1903) combined investigation of the juice with absorption experiments and concluded that cane-sugar was largely inverted after absorption, during its passage through the mucosa. Apart from some leucocytes, the only place in the body where invertase is found is the intestinal epithelium, but it seems likely that the enzyme is not secreted from the cells into the juice.

*Maltase.* Brown and Heron (1879–80) described the splitting of maltose by intestinal extracts and this has been repeatedly confirmed. Shore and Tebb (1892) and Tebb (1894) demonstrated also that maltase was found in a great variety of animal tissues in contrast with the restricted occurrence of invertase and lactase. Osato (1920) found the greatest concentration of maltase in the jejunum, less in the duodenum and still less in the ileum, while the colon had only a slight activity. He also pointed out that carnivores had a relatively high activity throughout the gut but in the herbivores the concentration decreased rapidly and in the ileum was very small.

In the *succus entericus* Röhmann (1887), Mendel (1896) and others reported activity, often slight. Bierry (1912) freed the juice of cells and microörganisms and still found some maltose. Röhmann and Nagano (1903) found that maltose was split by intestinal juice, but not so fast as it was absorbed by the mucosa.

*Lactase.* Röhmann and Lappe (1895) reported the presence of a lactose splitting enzyme in intestinal mucosal extracts. This was confirmed by Weinland (1899 and 1900), who advanced some evidence that its presence was restricted to young or growing mammals. Plimmer (1906–07) found it consistently present in the mucosa of young mammals of several species and persistent throughout life in many of them, whether or not they were kept on a milk diet. In an extensive series of experiments he found it impossible to restore lactase to the mucosa where it was absent, or to increase the amount present, by feeding large quantities of lactose. Several authors have reported lactase in *succus entericus* (e.g. Röhmann and Nagano, 1903; Savich, 1917), but the amount was always small and there were many negative results. Cajori

(1935), from experiments on centrifuged juice, came to the conclusion that lactase was not secreted.

The juice from Thiry-Vella fistulae did not yield appreciable amounts of *nuclease* (Levene and London, 1929) though gastro-intestinal fistulae gave juice which hydrolysed nucleic acid but not nucleosides (Levene and Dillon, 1930).

It is doubtful whether *arginase* is present in the intestinal mucosa (Oppenheimer, 1936, p. 578); it does not seem to have been looked for in the juice.

*Summarizing:* The *succus entericus* has significant and varied digestive powers. The amount of digestion by invertase, lactase, peptidase and lipase appears to run parallel with the cell content of the juice, suggesting that these enzymes are derived from cast off epithelial cells rather than from the mucosa by secretion. The probability is that they exert the most important part of their digestive action during absorption; Starling (1911, p. 127) mentioned that such a view was held by many physiologists, and Howell adopted it in his textbook (1926). The position of maltase is less certain. Reports on the relation of amylase to cell content are contradictory, but it has been so consistently found that its presence in the secretion must be considered likely, at any rate in the dog. Enterokinase has been found by all who have looked for it and is probably the most important of the enzymes. Rona and Weber (1927) and O. Kestner (1930) discuss these and other points associated with intestinal secretion.

It should be kept in mind that almost all the work has been done on dogs, and that species differences are possible. Human intestinal juice contained several enzymes after long and vigorous centrifuging (Hermann and Ribère, 1931) and was found by Hamburger and Hekma (1902 and 1904) to be equally active before and after filtration; in spite of the special fallacies of collecting material from human subjects this is a striking and unusual result. Juice from very thoroughly washed loops of human intestine contained small or moderate amounts of invertase, peptidase and lipase (Owles, 1937–38 b); the enzyme activity was said to be reduced by centrifuging, but comparative figures were not given.

THE CONTROL OF SECRETION. *Local stimulation.* The local stimulation of a fistula has already been discussed. Local stimulation has been applied by Owles (1937–38 c) to a loop of intestine in continuity, isolated by balloons. In response to hypertonic solutions there was a considerable output of fluid; air or normal saline had little or no effect on the volume produced.

*The influence of extrinsic nerves: The vagus.* There appears to be only one series of experiments in which vagal stimulation produced intestinal secretion. Savitch and Soshestvensky (1917) stimulated the vagi in the neck of decapitate cats and found that, after a latent period of 1 to 1½ hours, they could milk juice out of the small intestine at intervals. Their method of squeezing out the juice, producing as it does a local mechanical stimulation, was highly undesirable, but considerable quantities of juice were collected, up to 18 cc. an hour. The long latent period is peculiar, but they explained it by supposing that the vagus carried inhibitory as well as secretory fibers. Ten milligrams of atropine inhibited the secretion for ½ hour. It is of interest that no anesthetics were used in these experiments, since they have been shown (in the duodenum) to inhibit the secretory response to vagal stimulation.

In the experiments of Wright *et al.* (1940) stimulation of the vagus in cats caused secretion from the upper part of the duodenum, the mucoid constituent of the juice so produced coming from the Brunner's gland area. A small amount of watery juice came from the part of the duodenum just distal to the Brunner's glands but in no experiment was secretion elicited from the rest of the small intestine, though the stimulus was always adequate to produce secretion from the stomach and upper duodenum. In two experiments stimulation of the vagus produced a secretion from the intestine after the splanchnics had been cut in the thorax (cutting the splanchnics alone did not cause secretion).

There is thus not enough evidence to enable one to say definitely what influence the vagus has on the secretion of the small intestine. Possibly the right experimental conditions have not yet been devised.

*The sympathetic.* The influence of the extrinsic nerves on intestinal secretion first received attention in the middle of last century when in 1859 Bernard reported that the removal of the solar ganglion caused fluid to be produced in the intestine. Moreau (1868) investigated further and found that if he cut the mesenteric nerves to a loop of intestine it filled with fluid. This observation was many times repeated (Radziejewski, 1870; Hanau, 1886; Leubuscher and Tecklenburg, 1894; Mendel, 1896; Falloise, 1904; Molnár, 1909; Wright *et al.*, 1940).

A committee of the British Association (Pye-Smith *et al.*, 1874; Brunton and Pye-Smith, 1875 and 1876) performed experiments from which they concluded that the nervous centers which controlled intestinal secretion lay in the small ganglia of the solar and superior mesenteric plexuses, since they found that secretion was unaffected by cutting the splanchnics, the vagi, or the dorso-lumbar part of the cord. Recently the subject was reëxamined (Wright *et al.*) on decerebrate and

decapitate cats, with the following results: cutting the greater splanchnic nerves in the thorax caused secretion from the duodenum but not from any other level of the small intestine; cutting all the preganglionic fibers was followed by secretion from all the small intestine; the ganglia of the solar plexus took on the power to inhibit secretion within a few days after the preganglionic fibers had been cut; this "paralytic" secretion was inhibited by atropine. Mitsuda (1924) thought that the extra-intestinal sympathetic inhibited the secretory glands chiefly through the intramural sympathetic ganglia, but his experiments were inadequate.

The question has been debated whether the "paralytic" fluid so produced does in fact represent a secretion or whether it is a transudate (Kühne, 1878; Vulpian, 1874; Leubuscher and Tecklenburg, 1894; Wertheimer, 1902; Starling, 1911, p. 122). The majority have considered it to be a true secretion, a view strengthened by the observation that the "paralytic" fluid from the Brunner's gland area (see section A) contains the mucin typical of that region.

The juice is usually turbid and yellowish and contains a variable amount of thick gray or white mucus. The specific gravity is reported to be about 1009, the alkalinity equivalent to 0.20 per cent $Na_2CO_3$; Falloise (1904) gives the soluble and insoluble salts as 0.66 per cent and the organic solids as 0.75 per cent. He tabulates the comparative analyses of fistula juice, paralytic juice and transudate. Of the enzymes tested, invertase, maltase, peptidase (Mendel, 1896; Falloise) and enterokinase (Falloise; Wertheimer) have been reported present, protease and lactase absent (Mendel, Falloise, Tecklenburg, 1894), lipase and amylase contradictory (Falloise, Tecklenburg, Wertheimer, Molnár, 1909). Lately peptidase and invertase were again found, and were attributed to the cells in the juice (Wright *et al.*, 1940).

Mendel and Falloise adduced the presence of ferments in the juice as evidence that it was a true secretion, not realizing that they might have been accidental inclusions. The mucin and bicarbonate content are probably the best indications that it is in fact a secretion. The recent confirmation (Wright *et al.*) of Hanau's suggestion that atropine inhibits the continued formation of the juice is also in favor of its being a true secretion. The suggestion made by Starling (1911, p. 122), that it arises as a result of the vaso-dilatation is unsupported by any evidence.

*The influence of drugs.* It has long been known that pilocarpine will produce a flow of thin fluid from the intestine (Masloff, 1882; Vella,

1888 a; Hamburger, 1895; Glinski, 1891; Schepowalnikow, 1899; Sawitsch, 1904); as secretion continues the enzyme content of the juice diminishes.

Wright *et al.* found that eserine alone, in sufficient dosage, caused a copious secretion from the whole length of the intestine, without any concurrent flow of tears, saliva or tracheal mucus, or sweating of the pads of the feet. The juice was fluid, yellow, turbid and mixed with a variable amount of white mucus and débris. The administration of acetyl-choline to an eserinized animal often resulted in severe damage to the mucosa with stripping of villi. Atropine inhibited the action of eserine and acetylcholine.

Histamine given subcutaneously was stated by Koskowski (1926) to produce secretion from a Thiry-Vella fistula in dogs, but his evidence is not impressive. Nechoroschew (1929), too, claimed that histamine caused secretion but his description is far from clear and the quantities he obtained were small. Berndt and Ravdin (1934) and Cajori (1933) reported that the secretion of a modified Thiry loop, collected by a Johnston (1932–33) catheter, was considerably augmented by histamine; the increase reached its maximum half an hour after the injection. Lim *et al.* (1922–23) found no effect on the human duodenum. Owles (1937–38) considered that histamine had no specific action on the secretion of *succus entericus* in man.

Certain other drugs are said to produce small intestinal secretion when injected parenterally. Komarow found that large doses of methylguanidin (5–7 mgm./kgm.) (1924 b), carnosin (20–40 mgm./kgm.) (1924 c) and carnitin (1926) caused a secretion from the intestine as well as the salivary, lachrymal, bronchial and gastric glands.

*The influence of hormones.* The first report of a hormonal stimulus to intestinal secretion came from Delezenne and Frouin (1904). They obtained an abundant flow of juice from lower duodenal and upper jejunal fistulae by the intravenous injection of secretin-containing preparations. Bottazzi and Gabrieli (1905–06) using very large amounts of duodenal and jejunal extracts (15–40 cc.) also obtained a secretion, but Foà (1908), repeating their work, found that the extracts killed many dogs and only occasionally produced juice. Frouin (1905) got a secretion by intravenous injections of the *succus entericus* itself. Brynk (1911) put 200 to 300 cc. of HCl of different strengths into the stomach without increasing the secretion from a Thiry-Vella fistula. Delezenne and Frouin had obtained such an increase, but believed that the stimulus to secretion came from the intestine and not from the

stomach; they showed that secretion could be produced from a lower duodenal or upper jejunal fistula (though not from an ileal fistula) by putting acid into a second high fistula in the same dog. Savich (1921), too, found that HCl in one fistula increased the secretion from another but only if the nerves to the fistulae had been cut; a denervated fistula secreted also in response to feeding; Savich concluded that the nerves inhibited secretion.

Brestkin and Savich (1927) concluded that some humoral mechanism for stimulation existed. Agren (1934) reported that highly purified secretin caused intestinal secretion but his methods of testing seem rather inadequate and the amounts of secretion produced were very small. Nasset and his collaborators in a series of papers (Nasset, 1938; Nasset *et al.* 1938; Schiffrin and Nasset, 1939) have described a new hormone, distinct from secretin, which they have named "enterocrinin." The hormone was obtained from intestinal mucosa by extracting with HCl and alcohol and precipitating with NaCl, then extracting with glacial acetic acid and precipitating with ether and picric acid. The product when injected intravenously increased the amount of juice which could be obtained from a fistula by a catheter. Whether the methods of assay adopted by Nasset and his collaborators were adequate it is difficult to say; no juice could be collected without concurrent mechanical stimulus and the reason for this, as well as the mechanism of enterocrinin production and action, require further elucidation.

There is thus a varied collection of stimuli which will produce a flow of fluid from the intestine, namely, local mechanical, chemical and electrical stimuli, feeding and the introduction of acid into the stomach and upper intestine, secretin and "enterocrinin," sympathectomy, pilocarpine, eserin, and possibly histamine and other "non specific" substances. Vagal stimulation has not been clearly proved to produce secretion. Atropine inhibits certain of the secretory effects. Except for "periodic" secretion, which it inhibits, the ingestion of food has not been found to have much effect on the secretion of a fistula, though J. W. Boldyreff (1928) stated that bones in the food increased the secretion in dogs. Brestkin and Savich (1927) noted an increased flow of juice from denervated fistulae after food.

One difficulty in evaluating conclusions is that the results from acute experiments and from experiments on fistula-bearing animals are different. Most fistulae are partially denervated, and their final denervation as the pedicle atrophies is a slow process, allowing time for autonomic and hormonal balance to be readjusted; for example, al-

though sympathectomy produces much juice in the acute experiment, whether or not the vagi are cut, there is no hypersecretion from a totally transplanted fistula; similarly, the hypersecretion which follows abdominal sympathectomy disappears in a few days. Clearly knowledge must progress further before a definite view can be expressed as to which are the important factors in the control of intestinal secretion in the intact animal. It is still more difficult to estimate how all the factors involved are integrated and the cloud which covers this aspect of intestinal physiology is perhaps the cause of the fog surrounding many aspects of the pathology of the intestine.

*Argentaffine and Paneth cells.* It has been mentioned that little is known of the products of these cytologically very easily recognized cells. Efforts have been made, especially with the argentaffine cells, to identify their products by histo-chemical means. The reader is referred to Lison's book (1936) and Jacobson's paper (1939) for a discussion of the argentaffine cells, and to the article by Patzelt (1936) for the Paneth cells.

*Functions of the succus entericus.* It has been generally accepted that the *succus entericus* has an important part to play in digestion by virtue of the enzymes which it contains. However, from a survey of the literature it is by no means certain that the evidence is strong enough to support this view. It seems likely that few of the enzymes are actually secreted but that most of them enter the juice, as it were incidentally, from the break-up of cells. There can be no doubt that there is a constant and considerable desquamation of epithelial cells into the lumen of the intestine, shown, for instance by the large number of mitotic figures in the crypts of Lieberkühn, and by the presence of cells in the juice from a fistula.

The balance of evidence is that juice obtained by experimental methods from the jejunum and ileum is an alkaline fluid which, like that from the duodenum, contains only two secreted digestive enzymes—amylase and enterokinase. There is no satisfactory evidence whether such experimental juice has the same properties as the *succus entericus* secreted during the passage of chyme along the intestine, but it may be assumed that during digestion a fluid is secreted which has other functions besides that of contributing enzymes. It is well recognized that water and salt solutions are very rapidly taken up from the small intestine, and we have shown in this paper that duodenal secretion itself can be absorbed from the jejunum. It may be necessary for a constant secretion of fluid to take place from the crypts of Lieberkühn to keep the food particles in suspension while they are attacked by the pancreatic enzymes, and as the products of digestion are absorbed, water and salts go with them. One may therefore envisage a circulation of fluid during active digestion, the secretion passing out from the crypts of

Lieberkühn into the intestinal lumen and back into the villi. (Wright *et al.*, 1940.)

C. CECUM AND COLON. *Anatomy.* The mammalian cecum and colon are lined by a mucous membrane composed of crypts but no villi. The epithelial lining of the crypts is composed of large numbers of goblet cells interspersed between clear protoplasmic cells of various shapes and staining properties. As in the small intestine, the supply of new cells to replace those desquamated at the surface comes from the depths of the crypts where mitoses are frequent.

The cecum and colon are organs in which water absorption, with consequent drying of the feces, appears, at least in carnivora, to be the chief function. In herbivora they are the site of considerable digestion by bacterial action, especially of cellulose.

The secretory activity of these organs has been investigated by chronic fistula and acute experiments.

*Secretion by a fistula: The collection of juice.* The amount of spontaneous secretion produced by colonic fistulae in dogs is very small, indeed usually nothing escapes, though a gradually increasing plug of inspissated mucus collects inside (Florey, 1930). Babkin states that Berlazki (1903) collected 0.03 cc. an hour from one dog and 0.12 from another.

As in the small intestine, local mechanical stimulation by a tube increased the amount of juice, e.g., from 0.03 cc. to 0.24 cc. an hour in Berlazki's dog. De Beer, Johnston and Wilson (1935) collected as much as 19 cc. in 2 hours. Russian workers (Babkin, 1928, p. 798 to 799) could obtain no evidenee that secretion was influenced by feeding and they concluded that it was almost independent of events in the rest of the alimentary canal and depended mainly on local stimulation.

*Composition of fistula juice.* The material collection from cecal and colonic fistulae varies from clumps of white mucus to an almost watery fluid. Typically it is viscous and opalescent, with mucus unevenly distributed through it. Its reaction is alkaline, equivalent to an 0.04332 (1) per cent solution of $Na_2CO_3$ (Babkin, 1928, p. 797). De Beer, Johnston and Wilson (1935) found from 85.8 to 93.3 milli-equivalents of $HCO_3$ per liter, in 3 estimations. The specific gravity was 1,06131 and the fluid part of the secretion contained 98.6 per cent water, 0.63 per cent organic and 0.68 per cent inorganic substances (Babkin, 1928, p. 797).

Vella (1888b) claimed that cecal and colonic juice contained amylase, invertase and a proteolytic enzyme. Klug and Koreck (1883) in re-

porting their own findings—that secretion from the colon of dogs had no digestive action on starch, fibrin or olive oil—confirmed the negative conclusions of Czerny and Latschenberger (1874) and Marckwald (1875). In cecal secretion Strashesko (1904) found amylase, maltase, invertase and peptidase but no lactase or protease. To this list Wakabayashi and Wohlegemuth (1911) added a weak lipase. Enterokinase has not been found by any investigator.

Maestrini (1916) introduced small sacs into the intestine and also studied the juice and mucosal extracts. He concluded that peptidase, amylase and lipase were present in the proximal colon of the dog but that lipase was absent in the sheep; the enzymes decreased towards the lower colon except that invertase was sometimes present throughout the organ.

Roger (1905) has described a coagulating mucinase in fecal matter, Ciaccio (1906) and Riva (1905) confirmed this work, but Harding (1934) was unable to detect the presence of this enzyme.

*Control of secretion. Local stimulation.* It is a familiar observation in patients that the secretion of mucus accompanies inflammation of the colon. An analysis in cats of the factors involved (Florey, 1930; Florey and Webb, 1931) showed that local stimulation with, for example, dilute mustard oil, caused a considerable secretion of thick mucoid fluid like egg-white. This secretion was not inhibited by atropine or cocaine. The local application of histamine (1/1000), peptone (20 per cent), acetyl-choline (1/1000) or adenosine (1 per cent) was ineffective in causing secretion. Lium (1939) found, however, that 1 per cent acetylcholine applied to colonic "patches" on the abdominal wall produced secretion and gross damage. Acids applied locally, drying, rubbing and heating were also effective local stimulants (Florey, 1930). The conclusion was that the mucus-producing cells (goblets) were directly stimulated by the irritant to discharge their secretion. The irritant appeared to act on some secretory mechanism in the cell which called for oxygen, for in perfusion experiments cyanide inhibited the discharge of goblet cells in response to local irritation (Florey, 1931). It has been noted that goblet cells grown in tissue culture appear to discharge their mucin (Florey, 1930).

*The influence of nerves.* The nervous control of colonic secretion has been reinvestigated in recent years mainly on acute preparations.

a. *N. erigentes.* (*Pelvic nerves.*) Florey and Drury (1928) in a short note stated that stimulation of the *N. erigentes* in the cat caused secretion from the colon. After further experiments Florey (1930) believed

that there was insufficient evidence for the first conclusion. However, Wright *et al.* (1938) improved the experimental methods considerably and produced consistent results. They showed that prolonged faradic stimulation of the peripheral ends of the cut *N. erigentes* caused a clear mucoid fluid to be secreted by the colon. In contrast with the small amounts reported from fistulae, as much as 55 cc. was collected on one occasion in 8 hours from the distal half of the colon, while in many cats the average rate was about 5 cc. an hour. It appeared that the better the nutritional state of the cat the better was the result. Secretion was closely related to stimulation, stopping when stimulation was stopped and starting again when it was restarted.

Stimulation of the central cut end of one *N. erigens*, the other being intact, caused reflex secretion. The reflex center was in the lumbar cord. Previous reports of reflex excitation of secretion were made by Hay (1883) and by Larson and Bargen (1933b). In the former's experiments the results were not very clear cut and apparently they were never confirmed. Larson and Bargen found that an isolated segment of colon showed an increased secretion at the time of defecation; it was almost certainly, however, deprived of its secretory nerve supply (*N. erigentes*), since the nerves travel along the colon in the muscle coat; the secretion possibly depended on release from sympathetic inhibition. (Larson and Bargen (1933a) reviewed the literature dealing with the distribution of the *N. erigentes* and vagi.)

Additional evidence of the influence of the cholinergic nerves on colonic secretion has been obtained by using drugs. Pilocarpine in large doses has long been known to excite colonic secretion (Heidenhain, 1883; Vella, 1888b; Majewski, 1894; Florey, 1930). Florey suggested that the secretion might be caused by the violence of the muscular action of the colonic wall, but this was subsequently disproved (Wright *et al.*, 1938). Acetyl choline given *sub cutem* to eserinized cats, or applied locally (Lium, 1939), also caused secretion of mucoid juice. Atropine inhibited the secretory action of both nerve stimulation and drugs. Eserine given subcutaneously increased the amount of secretion produced by stimulating the *N. erigentes.* Jones and White (1938) found that pilocarpine and eserine caused secretion when applied to the colonic mucosa of students.

Koskowski (1926) reported that histamine injected intravenously caused a slight colonic secretion.

Anesthetics, especially those of the barbiturate type, were found greatly to reduce or almost to abolish the secretory response (Wright *et al.*, 1938) (cf. section A, duodenal secretion).

b. *Sympathetic nerves.* Section of the sympathetic nerves did not cause a "paralytic" secretion, but if the sympathetic to the colon was stimulated concurrently with the *N. erigentes* the amount of juice was less than when the *N. erigentes* were stimulated alone. The inhibition might be a direct effect on the secreting cells or due to the vascular contraction accompanying sympathetic stimulation.

These observations in the experimental animal show how profoundly the autonomic nerves can affect colonic secretion, and they give some support to the clinical hypothesis that "mucous colitis" or nervous spasmo-myxorrhea can be caused by a derangement of the colonic nervous mechanism. Many writers, e.g., Wakefield and Mayo (1938), Bockus, Bank and Wilkinson (1928), Cawadias (1927), Lium and Porter (1939), even consider that there is clinical evidence of a relationship between the hyper-motility and hyper-secretion of at least one form of mucous colitis and abnormal psychical or emotional states. In this connection, however, certain observers (e.g., Hollander, 1927) have produced evidence that mucous secretion of the colon is excited by the ingestion of food substances to which the patient is allergic.

None of the studies on stimulation of the pre-optic and hypothalamic nuclei have been concerned with colonic secretion, but other pelvic nerve activity (vesical contraction) occurs on stimulating the anterior commissure and adjacent part of the septum, and gastric motility and secretion have been reported following the stimulation of the hypothalamus (Beattie, 1932; Beattie and Sheehan, 1934). The whole question of hypothalamic function is reviewed by Ranson and Magoun (1939).

*Characters of the nervous secretion.* The secretion described by Wright *et al.* (1938) was a slightly opalescent fluid of varying viscosity. Sometimes it was so viscous that 10 cc. would drop out of a test-tube in one lump, while at other times it was as fluid as water. Small amounts of tough opaque white material could usually be found floating in it. It often had a spermatic odor, an observation also made on fistula secretion (Babkin, 1928, p. 797). It was alkaline, having a pH of 8.3 to 8.4 on collection, rising to 9.1 to 9.2 when $CO_2$ had been lost. It contained a surprising amount of alkali; 1.0 cc. neutralized 0.4 to 0.6 cc. of N/10 HCl. Other constituents were: organic matter, 0.4 per cent (less in some juices); total inorganic solids, 0.95 per cent; chloride, 0.35 per cent; phosphate, 0.5 mgm. per cent; calcium, 2.0 mgm. per cent. The mucin of the juice was precipitable by acetic acid.

No enterokinase, invertase, polypeptidase, trypsin or lipase were found but dipeptidase and a trace of amylase were present. The

dipeptidase may easily have come from cells in the juice, as no efforts were made to get rid of them. The enzyme content of this acute juice does not correspond very closely with that reported for fistula juice, which is doubtless very rich in cells.

*Reabsorption.* Permanent fistulae contain a growing plug of inspissated mucus, but in acute experiments the secrction poured out by the colon, even if very mucoid, could apparently be reabsorbed completely, leaving no trace, as soon as the stimulus was withdrawn (Wright *et al.*, 1938). This capacity of the mucosa to absorb its own product re-opens the question whether the same cell that secretes can also absorb.

*Histological changes associated with secretion.* After secretion, whether produced by drugs, nerve stimulation or by the local application of irritants, the colon of the cat showed a characteristic picture. The cells lining the bottoms of the crypts tended to become flattened, in extreme instances like squames (after mustard oil), and those containing mucus were discharged. The cells on the free surface were relatively much less changed, sometimes even appearing unaffected by the stimulus. This surprising distribution of the exhausted cells, which was regularly seen, has not been explained (Florey, 1930; Florey and Webb, 1931; Florey, 1932; Wright *et al.*, 1938).

The cycle of events in a goblet cell would appear to be as follows. The earliest stage of mucin production, according to Duthie (1933), is associated with the formation of droplets (which can be colored by neutral red) in relation to the basal mitochondria. The droplets migrate into the region of the Golgi body where mucin, stainable by such selective stains as mucicarmine, is elaborated. The view that the Golgi body is involved in the synthesis of mucin was advanced also by Nassonov (1923), Bowen (1924) and Florey (1932). As mucin production continues the droplets compress the nucleus towards the base of the cell and fill out the cell to a "goblet" shape. The mucin may possibly sometimes be discharged in such a way that the cell collapses, producing a "small" cell which can be recognized between the ordinary epithelial cells. In a quick discharge, however (e.g., under the influence of mustard oil), the mucin passes through the free end of the cell, leaving a protoplasmic cell of the usual type with the nucleus not compressed. By careful staining droplets of mucin can usually be demonstrated in the Golgi body area of such cells. If the stimulus is now withdrawn the cell starts to reform mucin, ready to repeat the cycle. The regeneration of mucin by exhausted goblet cells appears to be fairly rapid; a colon

severely depleted of its mucin by mustard oil shows very considerable recovery in two days. A discussion of some of the histological changes, including observations by the older histologists, has been written by Patzelt (1936, p. 111).

The part, if any, which the protoplasmic cells of the colonic mucosa play in secretion is not known, but they certainly lose some of their protoplasm in the secretion accompanying experimental inflammation. It would be interesting to have an analysis of the action of cathartics bearing in mind the points recently brought out about control of colonic secretion.

*Excretion.* The colon is commonly credited with the ability to excrete calcium, phosphate and heavy metals, but Taylor and Fine (1930), Nicolaysen (1934), Welch *et al.* (1936), and Johnson (1937) found no evidence for the excretion of Ca or $PO_4$. Cowell (1937), however, concluded from an analysis of the feces that the rabbit colon could excrete calcium, and Cohn and Greenberg (1938), who used rats, showed that 3 per cent of "marked" radio-active phosphorus was excreted by the colon in 8 hours, while 20 per cent to 30 per cent went out in the urine. Youngburg (1937), who examined the intestinal mucosa and contents of rats kept on a phosphorus free diet, concluded that there was a considerable elimination of phosphorus compounds through the intestinal wall. Heupke (1931) could obtain no evidence in a separated colon that aniline dyes, potassium ferrocyanide, quinine or gallic acid were excreted, but traces of iodide, bromide and thiocyanate appeared to be. Bargen *et al.* (1929) reported that parenterally administered neoarsphenamine, mercury, mercurochrome and metaphen were not, and that bismuth sometimes was, passed into the colon, but Leitch (1936–37), reviewing the subject, could find no indisputable evidence for any excretion by the colon of these substances. Boggino (1931) obtained histological evidence that injected iron was excreted by the goblet cells in the guinea pig. Ramond and Popovici (1931) injected iron hyposulphite in rabbits and found that iron was excreted by the cecum and proximal colon; lead, copper, mercury, silver and bismuth also, though very toxic, were excreted in the same region.

*Functions of the colonic secretion.* Information about the herbivora appears to be very scanty and possibly useful knowledge might be obtained by a study of the comparative physiology of the colonic secretion. The following conclusions as to its possible functions are based for the most part on results from dogs and cats.

It is difficult to believe that the supposed enzymes in the colonic

secretion could perform any important function. For reasons discussed in section B it is doubtful whether any actual secretion of enzymes would be shown if adequate methods and cell free juice were used.

Mucus appears to be the substance of most interest. Even very mucoid juice can be apparently reabsorbed and this means that the colonic mucosa can maintain a layer of mucus on its surface and can vary the thickness by secretion or reabsorption, according to the need of the moment. The mucus no doubt lubricates the solid feces as they pass through the lower colon and also helps to bind together the fecal particles; for example, hydrokollag given by stomach tube to a starved cat was subsequently found rolled into masses in the colon and on section the masses proved to be formed of carbon held together by mucus (Florey, 1933). Probably mucus is not digested in the colon but since a small percentage of colonic mucin makes a very viscous solution, the body loses little protein by mucin secretion even when quite large quantities of "mucus" are voided. Our general ignorance, however, of the fate of mucins in the alimentary tract is profound.

If the feces contain irritant matter fluid is produced, either by the direct local stimulus or *via* nervous reflexes. This protects the mucosa in two ways: first, by diluting the irritant, and secondly, by blocking its diffusion, for which a mucoid fluid is well suited. Evacuation *per anum* may then occur before the mucosa is seriously damaged. We have no exact idea what part nervous control plays in such a process nor how the nervous mechanism comes into play under natural physiological conditions. One can only register astonishment at the quantity of fluid that a cat's colon can produce when its secretory nerves are maximally stimulated.

The alkali content of the secretion is considerable, and probably has the function of neutralizing acids produced by bacterial action. For example, the interior of fecal masses from the cat's colon had a pH as low as 4.8 but at the surface the pH was 7 (Wright *et al.*, 1938). Tisdall and Brown (1924) found that the pH of infant's feces varied between 4.7 and 8.3, but in adults, according to Robinson (1922), it was 7 to 7.5.

*To sum up*, there is a large body of experimental work on secretion by the intestine, from the pylorus to the rectum, and this work has succeeded in analyzing some of the controlling factors. But we know little of how these factors are integrated to supply normal physiological needs; still less do we know in what way their integration differs from the normal in disease. The chronic preparation adapts itself to interference which in the acute preparation produces marked changes and it is this

adaptability, and the delicate balance of controlling mechanisms, which are so hard to investigate.

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# THE SIGNIFICANCE OF HYPERSENSITIVITY IN INFECTIONS

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When a foreign protein, whether bacterial or non-bacterial in origin, enters the tissues by whatever route, there ordinarily occur in the body certain alterations which affect the reactivity of the tissues toward subsequent contact with the protein. When these alterations have occurred, the body is said to have become "hypersensitive" to the foreign protein, for the tissues will then be readily injured by amounts of the protein that are entirely harmless to the normal body. Since all pathogenic microörganisms contain protein, an infected body becomes, as a rule, sensitized to the proteins of the microörganisms which invade the tissues. These proteins may be quite harmless to the normal body, but when the state of hypersensitivity has been established as a result of infection, minute amounts of the proteins will cause local damage and death of tissue, accompanied by acute inflammation; and marked constitutional symptoms and even death will occur when amounts of the proteins which are entirely harmless for the normal body enter the blood stream of the sensitized one. Even bacteria which have little or no power to kill the tissues of the normal body when injected locally, or to produce constitutional symptoms when injected into the blood stream, will exert both of these effects to a marked degree when introduced into the body that has been sensitized by previous contact with the bacteria. Since small numbers of bacteria, or minute amounts of their proteins, suffice to incite hypersensitive reactions in the sensitized body, it is obvious that the hypersensitive state is a factor that has seriously to be reckoned with in the interpretation of the lesions and symptoms of infectious diseases.

Differences between bacterial hypersensitivity and hypersensitivity of the Arthus type. It is important at the outset to stress the fact that there are certain differences between the protein hypersensitivity that is established as a result of contact of the tissues with microörganisms, and that which results from contact of the tissues

with a free foreign protein. When a free foreign protein, whether bacterial or non-bacterial in origin, is injected into the tissues, there develops a form of hypersensitivity which has, among others, the following characteristics: 1, the local or constitutional reaction produced by subsequent contact with the protein develops rapidly and reaches its height shortly after the test injection; 2, smooth muscle of the sensitized body is thrown into spasmodic contraction on contact with the specific protein; and 3, the hypersensitive state is transferrable passively to a normal body by injecting into it the serum of the hypersensitive one, i.e., sensitizing antibody is present in the blood stream of the hypersensitive body. This is the "anaphylactic" type of sensitivity. If repeated injections of the protein are made into the tissues at intervals, the local reaction increases in intensity with each injection until, finally, instead of eliciting merely a rapidly appearing and evanescent edema and erythema, such as characterize the local reaction of the anaphylactic state established by a single injection of the protein, a large, edematous, hemorrhagic and necrotic reaction will occur. This intensified local anaphylactic sensitivity is known as the "Arthus type" of hypersensitivity. As in the case of the local reaction in simple anaphylactic hypersensitivity, the onset of the Arthus reaction becomes macroscopically apparent promptly after the introduction of the protein into the tissues, and the hypersensitive state is transferrable passively.

While anaphylactic hypersensitivity to the bacterial protein may develop during infection, the characteristic state of protein hypersensitivity produced by contact of the tissues with microörganisms is different from either the simple anaphylactic or the Arthus type of hypersensitivity, and is ordinarily termed "bacterial hypersensitivity," "hypersensitivity of infection," or "tuberculin type hypersensitivity." The necrotizing inflammatory reaction produced by contact of the sensitized tissues with the specific bacteria or their extracted protein may, in its fully developed state, be indistinguishable in appearance from an Arthus reaction, but the state of bacterial hypersensitivity differs from tho Arthus type of hypersensitivity in the following ways: 1, the local or constitutional reaction resulting from contact with the bacteria or their proteins develops slowly, requiring some hours before it becomes obvious, in contrast to the prompt appearance of the reaction in the Arthus sensitized body; 2, the smooth muscle of the sensitized body is not thrown into spasmodic contraction by contact with the protein; and 3, the hypersensitive state is not transferable passively.

Exceptions to these and to other differences between bacterial and

Arthus type hypersensitivity may be encountered. For example, occasional, though irregular and unreproducible passive transfer of bacterial hypersensitivity has been recorded (1); and a transitory state of tissue reactivity of the slowly developing type may precede the appearance of anaphylactic sensitivity following the injection of certain foreign proteins into certain animals (2) (3). Exceptions such as these have led some investigators to believe that bacterial and Arthus hypersensitivity are states that are fundamentally the same; and this may, indeed, be the case. Most students of the matter, however, prefer to separate the two states until further information is available, for the above-mentioned differences are striking, and are the rule; and there are additional and no less striking differences. For example, the state of hypersensitivity that results from contact of the tissues with bacteria, whether living or dead, confers upon the body the ability to react hypersensitively not only to the protein derived from the bacteria, but also to the injection of the intact bacteria themselves. In contrast, the body that is brought into the state of Arthus hypersensitivity by the repeated injection of the protein derived from a given bacterium will react violently to the injection of that protein, but will not react hypersensitively to the injection of the intact bacteria (4) (5). We cannot enter here into a discussion of these differences, but it may be remarked that the failure properly to appreciate them has led to considerable confusion, and to certain erroneous conclusions that will be mentioned below.

DIFFERENCES BETWEEN THE MECHANISM OF THE TISSUE DAMAGE IN BACTERIAL AND IN ARTHUS HYPERSENSITIVITY. An important difference between the Arthus and the bacterial type of hypersensitivity lies in the site of sensitivity in the two states. In 1922, Holst (87) made a pioneering attempt to learn whether washed cells from the tuberculous body are hypersensitive to tuberculoprotein *in vitro*, but he stated that he was unable to reach any satisfactory conclusion by the technique employed. Rich and Lewis (6), using the tissue culture technique, were subsequently able to show that when tissue or blood cells of the body with bacterial hypersensitivity are removed from the body and well washed, they are killed *in vitro* by amounts of the bacterial protein which are innocuous to the cells of the normal body under the same conditions. This was confirmed by Aronson (7) and by Moen and Swift (8). Barg (9) and Aronson (13) found that the cells of the Arthus sensitized body, in contrast, can thrive *in vitro* in contact with the

specific protein[1]. Rich, Lewis and Gay (14) have confirmed this observation, and have further shown that the cells from human beings with asthma and hay fever, who were extremely sensitive to the specific antigen, were unharmed by contact with the antigen in tissue cultures. It is, indeed, impressive to see the cells of the Arthus sensitized body growing unharmed in tissue culture while bathed in an amount of the foreign protein that produces a necrotic lesion when injected into the body from which the cells were derived.

This, then, appears to be a fundamental difference between the hypersensitivity of infection and the Arthus type of hypersensitivity: in the former, the local tissue damage produced by the protein is due in large part to sensitization of the individual tissue cells; in the latter there is no generalized tissue hypersensitivity. How, then, is necrosis of tissue produced in the Arthus reaction, if the tissue cells at large are not sensitized?

It was Opie's (15) opinion that the specific precipitate that is formed by the interaction of the foreign protein with the precipitin of the sensitized body is a toxic substance that is responsible, in part at least, for the cellular damage that characterizes the Arthus reaction. The precipitate does appear to attract leucocytes when injected into the tissues of the normal body, but it does not cause necrosis (15); and in the tissue culture studies of Aronson (13) and of Rich, Lewis and Gay (14) the cells of the Arthus sensitized body were unharmed by contact with the specific precipitate. It is therefore unlikely that specific precipitate is directly responsible for the death of extravascular tissues in the Arthus reaction.

It has long been observed that vascular damage is a prominent feature of the histology of the Arthus reaction. Arthus (16), himself, noted the hemorrhagic character of the reaction; Opie (15), Gerlach (17), Laporte (30), Pagel (36) and others have well described histologically the vascular damage and thrombosis at the site of the reaction; and Abell and Schenck (18) observed these effects directly in the living rabbit's ear.

[1] Mendeléeff (11) and Sereni and Garofolini (12) have stated that the cells of the anaphylactic body are damaged *in vitro* by contact with the specific protein. The former writer makes only a very brief statement about the matter, and the experiments of the latter investigators are vitiated by the fact that the cells of the sensitized body failed to migrate and grow in the plasma of the same body even when the foreign protein was not added to the cultures. Neither Holst (87) nor Meyer and Loewenthal (10) could detect any damage caused by contact of the cells of the anaphylactic body with the specific protein *in vitro*.

The prominence of this vascular damage has suggested that the resulting interference with nutrition is an important factor in producing the tissue damage. Extending the above mentioned tissue culture studies to the intact body, Rich and Follis (19) have shown that whereas, in the Arthus sensitized body, tissues that contain blood vessels are readily killed by contact with the specific protein, the cells of a normally avascular tissue (cornea) are not killed by such contact[2]. If, however, the cornea is vascularized before the test injection is made, there occurs an Arthus reaction, with thrombosis of the newly formed capillaries, necrosis of their walls and hemorrhage.

The evidence derived from the *in vitro* and *in vivo* studies, therefore, indicates that in bacterial hypersensitivity there exists a type of sensitization of tissue cells which causes their death on contact with the specific bacterial protein, while in the Arthus type of hypersensitivity no such sensitization of the extra-vascular tissue cells exists. In the latter type of hypersensitivity, however, vascular damage does occur as a result of contact with the specific protein, and the surrounding tissue damage appears to be due to interference with nutrition resulting from the vascular damage and from the clogging of the tissue spaces with hemorrhage and exudate.

IS BACTERIAL HYPERSENSITIVITY DEPENDENT UPON ANTIBODY? There is no doubt that the simple anaphylactic and the Arthus type of hypersensitivity depend upon the presence of antibody. The fact that the injection of the serum of the sensitized body into a normal one confers sensitivity upon the latter is plain enough evidence of this. As was mentioned above, however, bacterial hypersensitivity is not transferable passively, and in this type of hypersensitivity no one has been able convincingly to demonstrate sensitizing antibody by any method. There are, nevertheless, a number of persuasive reasons for believing that bacterial hypersensitivity depends upon specific antibody.

To begin with, the fact that the antibody is not present in the circulation in an amount sufficient to permit passive transfer is no important argument against the view that this type of hypersensitivity is determined by antibody, for it is well known that even in anaphylactic hypersensitivity, in which sensitizing antibody is readily demonstrable in the serum, the increment of antibody that circulates in the blood stream is an excess which is entirely unnecessary for the hypersensitive reaction. The effective antibody is that portion which is intimately associated with the cells, whether actually attached to them or present in the im-

[2] It may be stated that when antibodies are present in the blood they penetrate into the normal cornea (20) (21).

mediately adjacent tissue fluids. Smooth muscle of the sensitized body, washed free of blood, contracts spasmodically on contact with the specific protein, and anaphylactic sensitivity may persist for a time after the circulating antibody has fallen to so low a level as to be indetectable by the usual tests.

One of the most persuasive reasons for believing that antibody is concerned in bacterial hypersensitivity is the high degree of specificity of the phenomenon—a degree of specificity that is difficult to account for on any basis other than as an antigen-antibody reaction. While it is true that cross reactions occur in certain instances (22) (due, presumably, to chemical similarities between the proteins of two different types of bacteria), these are exceptions; and the local reactions produced by the heterologous bacteria are usually less strong and frequently less typical than those produced by the bacterium that was responsible for the establishment of sensitivity. These cross reactions do not in the least disturb the probability that the state of hypersensitivity is dependent upon a specific antibody, for cross reactions are familiar even in the case of serological reactions that are definitely dependent upon specific antibody (cf. the agglutination of the proteus bacillus by the serum of patients infected with the Rickettsia of typhus fever).

In further support of the view that antibody is concerned in bacterial hypersensitivity it is pertinent to mention the anamnestic reaction. When a specific antibody has once been produced as a result of contact of the tissues with a foreign protein, the amount of antibody in the circulation gradually decreases with the passing of time until, at length, none at all can be detected. If, now, the antigen enters the tissues again, the specific antibody will reappear in the circulation, and will reach a given level in a much shorter time than was required following the first contact with the antigen. It was recognized long ago by von Pirquet (23) that when anaphylactic hypersensitivity, which depends upon antibody, has once been established it will gradually wane if there is no further contact with the antigen; but when the hypersensitivity has waned, the re-introduction of the antigen into the body causes the specific antibody and the hypersensitive state to appear again in a decidedly shorter time than was required following the first contact of the body with the antigen. It is significant, therefore, that this anamnestic reaction, which is so characteristic of antibody formation, is also a characteristic of bacterial hypersensitivity. Bacterial hypersensitivity declines to a very low level with the passing of time after an infection, but a fresh contact of the tissues with the specific bacteria will

cause the hypersensitivity to return to a high level in a shorter time than was required for its establishment following the first infection (24) (25). This will be discussed further, below.

Additional evidence that bacterial hypersensitivity is dependent upon antibody is contained in the phenomenon of specific desensitization. In the forms of hypersensitivity (anaphylactic, Arthus) known to be dependent upon antibody, the state of sensitivity can be greatly depressed or abolished by the proper injection of the specific protein to which the body is sensitized. It is significant, therefore, that bacterial hypersensitivity likewise can be depressed or abolished by desensitization, and that the same desensitizing procedures that are most effective toward this end in the case of the hypersensitive states known to be dependent upon antibody are precisely those that are most effective in ridding the body of bacterial hypersensitivity.

These considerations (and others could be cited) may suffice to show that there are close parallelisms between bacterial hypersensitivity and the forms of hypersensitivity known to be dependent upon antibody. It seems altogether probable that bacterial hypersensitivity is likewise dependent upon antibody, but that the antibody is closely bound to the cells, as indicated by the above cited *in vitro* experiments, and that too little excess antibody accumulates in the circulation to permit passive transfer.

THE NATURE OF THE SENSITIZING ANTIGEN. In the overwhelming majority of the instances in which anaphylactic hypersensitivity has been observed, the sensitizing antigen has been a protein. Attempts to induce hypersensitivity to substances other than proteins have so rarely been successful that it is clear that the animal body has little tendency to become sensitized as a result of contact with most non-protein substances. Indeed, the infrequency of sensitization to non-protein substances, and the almost universal sensitizing capacity of proteins, has led to the suspicion that proteins may be implicated in some way even in the instances of sensitization to non-protein substances. This possibility has been strengthened through the work of Landsteiner (37) and others, who have shown that non-protein substances of many types which are not antigenic in themselves may become so if they are united to proteins. The addition of such substances to a protein molecule can alter the antigenic specificity of the protein in such a way that when hypersensitivity is produced by the new molecule the body will be specifically sensitized to the non-protein component of the molecule, and will react hypersensitively to that component in

its native state, unattached to protein. It has been shown by Klopstock and Selter (38) and others that a non-protein, non-antigenic substance may unite with the protein of the blood if simply mixed with the serum *in vitro*, and that the new molecule has then the power to incite the formation of antibody specific for the non-protein component. It is altogether possible, therefore, that when the body becomes sensitized to non-protein substances such as carbohydrates or drugs, the sensitization may result from the union of a non-antigenic substance with the plasma or tissue protein, thus forming a new and "foreign" protein molecule. The matter is certainly not a settled one, however, and it must at present remain a question whether non-protein substances that induce antibody formation must attach themselves to body protein before becoming antigenic.

In the body sensitized by infection, it is only the protein fraction of the bacteria that is capable of eliciting the tuberculin type of reaction characteristic of bacterial hypersensitivity. The body may become sensitized to the bacterial polysaccharide during infection, but the hypersensitivity to the polysaccharide is always of the anaphylactic typo (39) (40) (86). It is important to note that whilo the extracted bacterial proteins are active in eliciting tuberculin type reactions in the body sensitized by infection, the injection of these proteins into the normal body has so far produced only the anaphylactic or Arthus type of sensitization. It is true that there are reports claiming the establishment of the bacterial type of sensitivity as a result of the injection of bacterial proteins, but those reports are not accompanied by evidence that the hypersensitivity so produced actually satisfied the criteria of bacterial hypersensitivity. Whenever proper study of the type of hypersensitivity produced by the injection of bacterial proteins has been made, the sensitivity has been found to conform to the anaphylactic or Arthus rather than to the bacterial type (41) (42). It appears, therefore, that the true sensitizing antigen responsible for bacterial hypersensitivity has not yet been isolated from any bacterium. The very interesting work of Dienes and Simon (2), Simon (142) and others (3) has shown that a transient state of cutaneous reactivity resembling bacterial hypersensitivity may appear several days after the injection of certain non-bacterial proteins into certain animals, disappearing as anaphylactic sensitivity appears. If that state proves to be actually identical with bacterial hypersensitivity, and if it can be produced by extracted bacterial proteins, those proteins would have to be regarded as representing, perhaps, the true sensitizing antigens of bacterial

hypersensitivity; but if that be so, then some additional factor associated with bacteria must be necessary for the persistence of the sensitivity that results from contact of the tissue with either living or dead bacteria.

The role of hypersensitivity in the production of symptoms. The symptoms that occur during infections may be caused by a variety of factors (toxic products derived from the microörganisms and from necrotic tissue; disturbance of the function of various organs; hypersensitivity) and it is frequently impossible to determine just to what degree each separate factor is responsible for the observed symptoms. This is rendered particularly difficult by the fact that each of several different factors may have the power to cause a single given symptom. Fever, for example, can be caused by toxic bacterial products, by products of necrotic tissue, by hypersensitivity to bland bacterial products. It is therefore difficult to separate the effects of hypersensitivity from those caused by other factors occurring during the progress of natural infection. It is, nevertheless, clear that the hypersensitive state can be responsible for a large part of the symptomatology that characterizes infections. This is brought out with particular clarity in the symptoms that occur during hypersensitive reactions to bland, non-bacterial foreign proteins which do not produce symptoms in the non-hypersensitive body. Serum sickness provides a familiar example of this. Horse serum injected into a normal human being produces no appreciable symptoms, but after the lapse of a week or so, when the body has developed antibodies to the proteins of the foreign serum and is thereby rendered hypersensitive, if some of the serum still remains in the circulation, or if a further injection of serum be made, there will occur symptoms which are strikingly like those common to many infections (fever, malaise, aching joints and back, headache, anorexia, prostration). Likewise, when the body has been sensitized to bacterial proteins as a result of infection, the subsequent injection of small amounts of those proteins after recovery from the infection will cause symptoms of the above-mentioned character. As a familiar example, the proteins of the tubercle bacillus can be injected into the normal body with no resulting symptoms, but if hypersensitivity has been established by a slight and completely resisted tuberculous infection, the injection of minute amounts of the bacillary proteins will produce the train of symptoms described above. Likewise, in the animal sensitized by infection, high fever and severe constitutional symptoms will follow the injection of a number of intact tubercle bacilli which will cause no appreciable

symptoms in the normal animal. Furthermore, if the hypersensitive state be abolished by appropriate desensitizing procedures, amounts of tubercle bacilli or their extracted protein which would cause violent symptoms and death in the hypersensitive body can be injected with impunity. This is familiar in the therapeutic desensitization of tuberculous patients (135) (140), and can be readily demonstrated in experimental animals (26) (27). Years ago von Pirquet (23) drew attention to the analogy between the incubation period of infectious diseases and the latent period that extends from the time of injection of a foreign protein to the appearance of serum sickness. Since, in the latter case, the latent period is known to be the period during which hypersensitivity is becoming established, and the appearance of symptoms to be due to the action of the protein on the hypersensitive body, von Pirquet advanced the reasonable idea that in certain infections the symptoms that appear following the incubation period are due to the development of hypersensitivity. There are many reasons for believing that this is often the case, though in some instances the incubation period may be due, in part at least, to the time required for the invading microörganisms to multiply to numbers sufficient to damage the body by their own toxic products. However, since it is well established that relatively bland bacterial proteins are able to produce in the sensitized body symptoms that are quite like those resulting from the action of true bacterial toxins, it is clear that the hypersensitive state confers a potent toxic activity upon even natively harmless products of bacterial disintegration.

The influence of hypersensitivity on the character of the lesions. The hypersensitive state intensifies the degree of inflammation and the amount of tissue necrosis that a given number of bacteria or a given amount of their proteins will produce. These effects of hypersensitivity form the basis of numerous diagnostic tests, such as the tuberculin reaction, and are demonstrable in most acute and chronic infections produced by infecting agents of such widely different types as pyogenic bacteria, fungi, mycobacteria, spirochetes, Rickettsia and filterable viruses. In many cases it is impossible merely by looking at a lesion under the microscope to differentiate the effects of hypersensitivity from those of toxic products elaborated by the microörganisms, but in controlled experiment the influence of hypersensitivity in exaggerating inflammation and necrosis can be revealed in a striking way by comparing the lesions produced by a measured number of bacteria in the normal and in the hypersensitive body. Furthermore, if an estab-

lished hypersensitivity be reduced or abolished by desensitization, the amount of inflammation and necrosis that a given number of bacteria will produce in that body will be correspondingly lessened. This has been clearly demonstrated in both acute (28) and chronic (26) infections. As in the case of the influence of hypersensitivity on symptoms, it may be said that the hypersensitive state converts, in effect, even bland proteins of microörganisms into substances that are highly potent in their power to damage tissue.

It has been the experience of most investigators that except for the presence of eosinophils in some anaphylactic inflammations, there is nothing qualitatively specific about the inflammatory exudate produced by bacteria or their products in the hypersensitive body. Practically all who have studied the evolution of the hypersensitive inflammation have recorded the appearance of an exudate in the initial stage of which polymorphonuclear leucocytes predominate, with a gradual increase in mononuclear cells until, after a day or two, the latter cells are present in abundance (43) (44). This is the usual sequence in acute inflammation produced by irritants in the normal body. Dienes and Mallory (29), however, state that in their experience the inflammatory reaction of bacterial hypersensitivity differs from ordinary acute inflammation and from that of the Arthus reaction in that mononuclear cells predominate in the exudate at all stages from the very beginning; and that even in cutaneous tuberculin reactions involving necrosis of the epithelium polymorphonuclears are rarely predominant. Laporte (30), from a cytological study of tuberculin reactions, states that in weak reactions mononuclears predominate at all stages, but none of his descriptions of early reactions show that to have been the case, and in his conclusion he states that "one finds in all cases an afflux of polymorphonuclears in the first hours and a macrophage reaction later." Dr. R. H. Follis, Jr. (100), in this laboratory, has carefully repeated the technique of Dienes and Mallory and has been unable to confirm their report that mononuclear cells predominate from the beginning in mild and moderately intense tuberculin reactions.

Whether or not mononuclear cells ever actually predominate over polymorphonuclears in the initial stage of the reactions of bacterial hypersensitivity, it is true that mononuclears are often more abundant in early reactions of this type than in the early stages of acute inflammation produced in the normal body by irritants in general. To what extent this may be attributable to hypersensitivity requires further study. In tuberculosis it may be due, in part at least, to the well known

fact that the circulating monocytes are increased during infection, providing a correspondingly greater opportunity for more of these cells to escape at inflamed sites. It may be remarked that Dienes and Mallory (29), Laporte (30) and Lurie (45) noted that more mononuclear cells were present in inflammations produced by non-specific irritants in the tuberculous body than in the normal one. It should be possible to determine *in vitro* whether the mononuclear cells of the hypersensitive body are attracted by tuberculoprotein more strongly than are polymorphonuclear cells, and also, by means of tissue cultures, whether the multiplication of the mononuclears of the hypersensitive body is stimulated by amounts of tuberculoprotein too small to kill the cells.

Some writers (31) (32) state that hypersensitivity is responsible for the development of tubercles and tuberculoid tissue in tuberculosis and in the other infections in which tubercles occur. Others (33) (34), in direct contrast, insist that tubercles are necessary for the development of hypersensitivity. Neither of these generalizations can be accepted without reserve at present. It is true that, in response to a given number of bacilli, tubercle formation occurs more rapidly and more abundantly in the tuberculous body than in the normal one; but the view that the specific tuberculous lesions "represent the response of allergic tissue to the presence of bacteria, and are not produced by special properties of the tubercle bacillus" (32) is not tenable, for tubercles and tuberculoid tissue can be produced in the normal body by the injection of even the lipide extracted from the bacilli (35). Also, tubercles and tuberculoid tissue continue to develop in the tuberculous body that is kept desensitized to the degree that very large amounts of bacilli or tuberculoprotein fail to produce hypersensitive inflammation or necrosis (26) (27). Furthermore, in infections caused by many other types of bacteria the presence of hypersensitivity does not cause the formation of tubercles. As for the view that in tuberculosis "either the actual tubercle or a special type of tissue cellular reaction is essential for the establishment of the state of hypersensitivity" (34) this, too, seems unlikely, for bacterial hypersensitivity develops in the many other infections in which such lesions do not occur at all.

As stated above, the agglomeration of mononuclear phagocytes to form tubercles occurs more rapidly in the tuberculous than in the normal body at sites where bacilli lodge, and this has widely been regarded as a manifestation of specific hypersensitivity. There is, however, a lack of conclusive evidence for that interpretation; and while it may be

correct, certain circumstances relating to hypersensitive reactions warrant a critical attitude toward accelerated tubercle formation until its mechanism is clearly established. The existing evidence informs us that it is the bacillary protein which evokes the specific hypersensitive reaction, while only the lipide derivatives of the bacillus incite tubercle formation. Tubercles are not formed in hypersensitive reactions to tuberculoprotein, except non-specifically at sites where unabsorbed necrotic tissue (especially necrotic fat tissue) acts as a foreign body; and there is no convincing evidence that the tubercle-inciting lipides of the bacillus evoke specific hypersensitive reactions. Holley (129) noted that tuberculous animals responded to the injection of tuberculophosphatide with more inflammation than did normal animals, but he attributed that to the presence of tuberculoprotein in the preparation. Smithburn and Sabin (130) observed that a preparation of tuberculophosphatide (protein free?) caused larger reactions in the tuberculous than in the normal body. The microscopic difference between the reaction in the tuberculous and the normal body consisted, however, not in an acceleration or increase in tubercle formation, but in an increase in inflammatory exudate in which polymorphonuclear leucocytes were prominent. Smithburn and Sabin suggested that the reaction in the tuberculous body might be non-specific and similar to the intensified reaction that various non-specific irritants produce in the tuberculous body. Boquet and Nègre (131) were unable to produce hypersensitive reactions in the tuberculous body by the injection of tuberculophosphatide; and Long (132) was unable to detect any hypersensitive effect when various lipides of the bacillus were injected even into the highly sensitive testis of the hypersensitive body. Since the lipides constitute the only portion of the tubercle bacillus which has been found to stimulate tubercle formation, the more rapid development of epithelioid cells and tubercles in the immune, hypersensitive body may, as suggested by the observations of Lewandowski (123), Lurie (124) and others, be due to the immunity rather than to hypersensitivity, for acquired immunity confers upon the body an enhanced ability to destroy the bacilli and thereby to liberate their tubercle-provoking lipides. These considerations render desirable further studies of the mechanism of accelerated tubercle formation.

Many writers have sought to attribute to hypersensitivity a wide variety of lesions of as yet undetermined pathogenesis (e.g., nephritis, the lesions of rheumatic fever, lobar pneumonia). The arguments so far brought forward have been, at best, only suggestive, and they

cannot be analyzed fairly in the space of the present review. Continued investigation of the rôle of hypersensitivity in such conditions is altogether desirable.

THE ROLE OF HYPERSENSITIVITY IN RESISTANCE TO INFECTION. During infection with most microörganisms the body usually develops an increased ability to protect itself against the attack of the specific invader. Aside from the production of antitoxins which serve to neutralize toxic products liberated from the bacteria, this acquired resistance manifests itself in two prime ways. First, the spread of bacteria from the place where they lodge is inhibited; and second, their multiplication is restricted, and many or all of them are destroyed. By the side of this acquired resistance to infection there develops the "bacterial type" of hypersensitivity to the protein of the microörganisms. In the early part of this century, the dramatic manifestations of hypersensitivity observed in the experimental animal that had developed acquired resistance during tuberculous infection, together with the failure of attempts to demonstrate the protective activity of the antibodies formed during tuberculous infection, led to the widespread belief that the hypersensitive state is responsible for the increased resistance acquired during infection; and the view that hypersensitivity is an essential or highly important mechanism of immunity was then extended to include infections in general. While the destructive effects of hypersensitive reactions have always been clearly recognized, they have been regarded as necessary evils which had to be borne for the protection of the body as a whole, for it has been assumed that the inhibition of spread of bacteria in the immunized body is accomplished by the mechanical, walling-off effects of the accelerated and exaggerated hypersensitive inflammation, and the exaggerated inflammation has also been assumed to be necessary for the more efficient destruction of bacteria which occurs in the immune body. In the space remaining in the present review it is impossible to treat thoroughly the question of the relation of hypersensitivity to immunity. The reader may consult Pagel's (36) recent review, and the discussions in the papers cited in the present article, and should certainly read the reviews of Opie (46) (48), who has favored the view that hypersensitivity is an important mechanism of immunity. In evaluating any writings on this subject it is advisable that the reader be on his guard against being misled by the lamentable and confusing lack of precision in the use of the terms relating to hypersensitivity and immunity. "Allergy," for example, is used by many only as a synonym for hypersensitivity; others

include in the term all manifestations of acquired immunity; and still others in a single paper, shift their meaning of "allergy" backward and forward from one to the other of the above concepts. On the other hand, the term "immunity" is often applied to states in which the body has been rendered susceptible to injury by a foreign substance rather than protected against it. For example, when hypersensitivity has been established to a bland foreign protein which has no power to injure the normal body, it is common that the investigator speaks of having "immunized" the body against the protein, though in reality he has rendered the body hypersusceptible to injury by it. In discussions of the problem whether increased susceptibility of the tissues to injury by a bacterium (hypersensitivity) is necessary for the protection of the body against that bacterium (immunity), ambiguities of the above-mentioned nature frequently confuse the issue completely.

The view that hypersensitivity is a necessary or highly important mechanism of acquired resistance has been based chiefly upon two premises: 1, that an accelerated and exaggerated hypersensitive inflammation inhibits the spread of bacteria (49) (51); and 2, that hypersensitivity and acquired resistance parallel each other (50). In no case has it been proved that acquired resistance cannot function as well in the absence of hypersensitivity as in its presence. Let us proceed to examine the two above-mentioned generalizations.

*Is hypersensitive inflammation the mechanism responsible for the inhibition of spread of bacteria in the immune body?* There is no doubt that after inflammation has become well established the exudate can form a mechanical barrier that will inhibit the further spread of bacteria; and it is also well known that the multiplication, survival and spread of certain bacteria are markedly inhibited if they are deposited in tissues or serous cavities which have been inflamed for some time before the bacteria are introduced (52) (53) (54) (49). This fact has led to the assumption that the rapidly developing hypersensitive inflammation is a mechanism responsible mechanically for the inhibition of spread of bacteria in the immune body (48) (51). It is important, however, to point out two facts. First, not all types of bacteria to which the body develops effective acquired immunity are restrained by an area of prepared acute inflammation (53) (55) (56); and second, and more important, even those types of bacteria which are restrained when deposited in a previously inflamed area are not restrained if they are introduced into the normal tissues of the non-immune body under conditions which ensure the development of a rapid hypersensitive

inflammation about them. A number of years ago the writer (58) pointed out that the experiment of depositing bacteria in an area that is inflamed *before* the bacteria are introduced does not at all reproduce what occurs in a hypersensitive reaction. In the latter case the bacteria are deposited in normal tissues, and the hypersensitive inflammation develops about them *after* they reach the tissues. Since bacteria are known to spread with great rapidity from the site where they lodge in the non-immune body (57), and since in the early stages of inflammation particulate matter tends to be spread from the site by the increased movement of tissue fluid, it seemed altogether unlikely that even in the hypersensitive body inflammation could develop rapidly enough in sufficient amount to account mechanically for the remarkable inhibition of spread of bacteria observed in the immunized body; and, indeed, the writer showed that when the time conditions obtaining in the hypersensitive reaction were reproduced by causing an accelerated hypersensitive inflammation to develop about bacteria in the previously normal tissues of a body not immune to the bacteria, the spread of the bacteria was accelerated rather than retarded, and in no case did the rapidly developing hypersensitive inflammation protect the animal from a fatal outcome, even though only minute numbers of bacteria were introduced (58). This result has been confirmed by other investigators, using a variety of different bacteria (59) (60) (61). In subsequent studies the writer (62) (63) showed that in the immunized body the immediate immobilization of bacteria is accomplished by the action of antibody, which causes the bacteria to adhere to each other and to the tissues where they lodge; and the immobilization was shown in passive transfer experiments to be altogether as effective in the complete absence of hypersensitivity as in its presence. This immobilizing action of antibody was confirmed by Cannon and Pacheco (64), Catron (65), Pickrell (153) and Wood (154); and Rich and McKee (66) demonstrated that it operates even in the complete absence of a cellular or fibrinous inflammatory exudate. These considerations render untenable the view that the inhibition of spread of bacteria in the immune body is dependent upon the mechanical walling-off effects of a rapid hypersensitive inflammation.

For many years the relation of bacterial hypersensitivity to immunity was studied almost exclusively in experimental tuberculosis in the guinea pig, and the view that hypersensitivity is necessary for the successful operation of acquired immunity has been based largely upon the fact that in that situation hypersensitivity and acquired resistance

usually parallel each other (50). Occasional clinical (67) (68) and experimental (69) (70) (71) investigators raised their voices against the doctrine that hypersensitivity and immunity necessarily parallel each other, and a number of years ago Rich and McCordock (72) assembled the then existing evidence against the view that hypersensitivity is necessary for immunity. Since then, numerous and weighty observations have been brought forward, demonstrating that acquired resistance does not depend upon hypersensitivity. This latter evidence will be briefly summarized.

*The separation of immunity from hypersensitivity by passive transfer.* Rich and Brown (73) showed that if the serum of animals that are hypersensitive and immune to the pneumococcus be injected intravenously into normal animals, the immunity will be transferred but not the hypersensitivity. In contrast to the large, necrotic hypersensitive reactions produced in the skin by a given number of pneumococci in the immune, hypersensitive animals, it was ordinarily almost impossible to see any lesion whatever macroscopically in the passively immunized animals, and microscopically only a trivial inflammation (far less at all stages than in either the normal or the hypersensitive animals) was found (63); and yet in the complete absence of a hypersensitive reaction the bacteria were sharply localized at the site, septicemia was prevented, and the animals rapidly overcame the infection which was invariably fatal to non-immune controls. Spared from the destructive effects of hypersensitivity, the passively immunized animals, by all tests, prevented the spread of the pneumococci and resisted the infection altogether as well as did the actively immunized hypersensitive ones, even when subjected to several million times the lethal dose for a non-immune animal.

*The lack of parallelism between immunity and hypersensitivity.* As stated above, the parallel development of immunity and hypersensitivity has provided one of the main arguments in favor of the belief that immunity is dependent upon hypersensitivity. The parallel development of these two states was, however, studied in practically no infection other than tuberculosis. From this limited information, and without further investigation, the parallelism of immunity and hypersensitivity was assumed to apply as a generalization that covers all infections. There is now convincing evidence that no such generalization is warranted. Even in infections in which hypersensitivity and immunity tend to develop concomitantly, their dissociation has been effected by appropriate methods of immunization. Thus, Swift and Derick (74) have shown that whereas animals immunized by the

injection of *non-hemolytic streptococci* into their tissues develop a high degree of hypersensitivity, the test reactions in animals immunized by repeated intravenous injections are less conspicuous even than those occurring in normal animals. This was confirmed by Clawson (75). Petroff, Branch and Jennings (101), Branch and Cuff (102) and Clawson (103) using the *tubercle bacillus*, found that intravenous immunization often failed to establish hypersensitivity. It is probable that the absence of hypersensitivity in these experiments was due, in part at least, to the desensitizing effect of the repeated intravenous injections of bacteria. In these studies, when resistance to infection was tested it was found to be as high in the immunized, non-hypersensitive animals as in hypersensitive ones. In the case of a chronic infection such as tuberculosis, it might be argued that even though the immunized animals had no hypersensitivity when they received the virulent test infection, hypersensitivity could have appeared after some days as a result of the infection itself. To this it need only be remarked that hypersensitivity can be demonstrated within a few days after the infection of a normal, non-immunized animal, and in this case the hypersensitivity does not prevent the devastating progress of the infection. Some years ago Rich and McCordock (72) presented evidence against the then current belief that immunity and hypersensitivity necessarily parallel each other in tuberculosis; and recently, Freund and Opie (104), from a careful study of the development of these two states in experimental tuberculosis, have concluded that "there is no correlation between intensity of sensitization . . . and resistance to infection." Indeed, Sewall, de Savitsch and Butler (105) concluded from their experiments on acquired immunity in tuberculosis that "*immunity is somewhat inversely proportional to the intensity of local allergic reactions*" (author's italics), and Boquet (127) likewise found that "the intensity of the local reaction diminishes as the immunity engendered by the first infection increases." Below we shall see that even when hypersensitivity is prevented from making its appearance during the course of the experiment following infection, immunized animals are protected against the tubercle bacillus in as high a degree as are hypersensitive ones.

While hypersensitivity has often been observed to appear during *pneumococcal* infection, Mackenzie (76) found that when guinea pigs were immunized by the intraperitoneal route they developed a high degree of acquired immunity, but they did not react hypersensitively to the intracutaneous injection of pneumococci or pneumococcal protein.

Numerous writers, without advancing evidence, have stated that

acquired immunity in *syphilis* is dependent upon a local hypersensitive reaction at the site of reinfection (77) (78) (33). Rich, Chesney and Turner (80) have made a detailed study of this point. Rabbits were immunized by intratesticular inoculation, and at various intervals following the immunizing inoculation they were reinfected intracutaneously with large numbers of virulent spirochetes. The sites of reinfection were carefully observed macroscopically, and were removed at daily and weekly intervals for microscopic study. Although the reinfecting dose of spirochetes was far larger than could ever occur in natural infection, in no case was there the slightest indication of a more prompt or exaggerated inflammatory reaction in the immunized animals as compared with control animals, and yet the former exhibited the characteristic high degree of immunity to the test infection.

The lack of parallelism between hypersensitivity and immunity has been noted in *hemolytic streptococcal* infection by Angevine (83), who found that the intracutaneous injection of an avirulent streptococcus produced a high degree of hypersensitivity with scant immunity, whereas a virulent strain produced the reverse effects. He concluded that "increase in virulence of hemolytic streptococci enhances the ability to protect against local infection and increases antibody formation, but diminishes the production of sensitization."

The lack of parallelism between immunity and hypersensitivity has been observed not only in bacterial and spirochetal infection, but also in *filterable virus infection*. In a study of immunity to the filterable virus of infectious myxomatosis McKee (143) found that hypersensitivity appeared in 7 to 14 days after a single vaccinating injection of killed virus, whereas acquired resistance developed later, and usually only after repeated immunizing injections. In animals infected during the period when sensitization was present without appreciable immunity, the local reaction was more severe and generalized lesions appeared earlier than even in normal controls.

Finally, in this connection, it may be pointed out that all investigators are agreed that there is no parallelism between the degree of hypersensitivity and the titer of protective antibodies. Indeed, in the instances studied so far, the antigen that stimulates the development of protective antibody has been found to be different from that to which the body develops bacterial hypersensitivity. Thus, in the case of the pneumococcus, whereas it is the protein portion of the bacterium to which bacterial hypersensitivity develops (39), it is the polysaccharide fraction that is the potent immunizing antigen (84) (85). Injection

of the purified polysaccharide produces a high degree of immunity against infection with the pneumococcus, and this immunity is not accompanied by the bacterial type of hypersensitivity. Anaphylactic sensitivity to the polysaccharides of bacteria may develop, but there is no evidence whatever that anaphylactic sensitivity plays any rôle in protection against infection. Animals immunized against the tubercle bacillus, for example, ordinarily do not respond with a prompt anaphylactic wheal and erythema to the intracutaneous injection of tubercle bacilli. As in the case of the pneumococcus, it is the polysaccharide that is the effective immunizing antigen of the Friedländer bacillus. Morris (86) passively immunized animals to the Friedländer bacillus with an immune serum that also rendered them anaphylactic to the polysaccharide of the bacillus. He then abolished the anaphylactic state by desensitizing injections of the polysaccharide, and demonstrated that immunity to infection remained intact in the absence of anaphylactic sensitivity. Since the animals were passively immunized they also lacked the bacterial type of hypersensitivity.

These various experiments (and many others could be cited) serve to illustrate the fact that not only is there no necessary parallelism between the degree of immunity and hypersensitivity, but also that acquired immunity can be established in widely different types of infections without the concomitant development of hypersensitivity, and that the immunity thus established is as effective in overcoming the infection as is immunity accompanied by hypersensitivity. Indeed. this situation has the decided advantage that the tissues are spared from the damaging effects of hypersensitivity while the microörganisms are being subjected to the forces of acquired immunity.

*The separation of immunity from hypersensitivity by desensitization.* The above studies on widely different types of infection made it clear that there is no necessary parallelism between hypersensitivity and immunity, and that acquired immunity can operate efficiently even in the complete absence of hypersensitive inflammation. Willis (24), Sewall, de Savitsch and Butler (105), Calmette (71) and others have shown that the hypersensitivity of immunized animals wanes with the passing of time, but immunity to infection remains intact. Since hypersensitivity appears again during the test infection in such experiments, and since hypersensitivity has often been assumed to be necessary for the operation of immunity in the stage of immunization in which it (hypersensitivity) is present in high degree, it remained to determine whether, in the immunized body in which bacterial hyper-

sensitivity is present in high degree, the maintained abolition of the hypersensitivity by desensitization would leave the immunity unimpaired. In order readily to ensure the persistence of the state of desensitization throughout the entire period of the test infection, Rich, Jennings and Downing (28) first chose acute infections (type I pneumococcus; Pasteurella avisceptica) which regularly run their course to a fatal termination in non-immune animals within a period of hours. Rabbits were rendered immune and hypersensitive to the respective bacteria by appropriate vaccination. Half of each series were then desensitized by intravenous injections of a heavy suspension of the killed bacteria. The desensitized animals, the undesensitized ones and normal controls were then infected intracutaneously with several million lethal doses of the living bacteria. The results were clear cut. The local inflammation produced by the living bacteria in the desensitized animals was at all periods far *less* in amount, both macroscopically and microscopically, than that in the undesensitized animals *or in the normal controls,* and the tissue damage was also far less; and yet immunity to millions of lethal doses of the highly virulent bacteria remained intact. Blood cultures and bacterial stains of the local lesions showed that the bacteria were as effectively prevented from spreading from the site of infection and were as effectively destroyed in the desensitized animals as in the hypersensitive ones. It was impressively clear that even in the highly hypersensitive body the accentuated inflammatory reaction of hypersensitivity is not necessary for protection against the infection.

These studies on desensitization in acute infections were extended to tuberculous infection by Rothschild and his co-workers (26) in this laboratory. In a carefully controlled study they showed that immunized, hypersensitive animals can, by appropriate treatment with tuberculin, be desensitized so that they no longer react hypersensitively with accelerated and exaggerated inflammation and necrosis to the local injection of large amounts of virulent tubercle bacilli or tuberculin, and they tolerate with no evident symptoms the subcutaneous or intraperitoneal injection of amounts of tuberculin that would invariably be fatal for hypersensitive animals. Animals thus desensitized, together with normal controls and animals that were immunized but left hypersensitive, were infected intracutaneously, subcutaneously and in the anterior chamber of the eye (a site highly favorable for the detection of small differences in inflammatory response) with a standard dose of virulent tubercle bacilli, and the desensitized animals continued to receive their large daily dose of tuberculin which maintained the state of

desensitization throughout the experiment. Just as in the case of the acute infections described above, it was found that the desensitized animals remained as highly resistant to the proliferation and invasion of the bacilli as were the immunized, hypersensitive ones; and the former were spared from the destructive local hypersensitive effects suffered by the hypersensitive animals at the sites of infection. At the time when the organs of the non-immune control animals were riddled with tuberculous lesions, the desensitized and the hypersensitive animals showed only the minimal lesions characteristic of immunized animals, and those lesions tended, if anything, to be less numerous and less necrotic in the desensitized than in the hypersensitive animals.

Following these experiments there appeared a succession of confirmatory studies (Siegl (88); Higginbotham (22); Birkhaug (82); Cummings and Delahant (79); Boquet (89); Selter and Weiland (91); Thayer (93); Balteanu, Toma and Garaguli (94); Saenz (136); Wilson, Schwabacher and Maier (137); Branch and Kropp (92); Corper (144); Derick, Branch and Crane (90); Follis (27)). In some of these studies hypersensitivity was profoundly depressed, but not completely abolished (79) (82) (89) (93) (136) (137). In other studies (26) (22) (88) (27) (92) desensitization was complete (absence of local or constitutional hypersensitive reaction to very large amounts of bacilli or tuberculin). In all of the studies the state of desensitization was maintained throughout the entire duration of the experiment. All of these studies confirmed the fact that the hypersensitivity of immunized animals can be markedly depressed or completely abolished with no loss of acquired resistance to virulent tuberculous infection. Indeed, in most of the reports the investigators state that the resistance of the desensitized animals to the test infection was greater than that of hypersensitive controls (22) (26) (82) (89) (93) (94) (137) (92).

Bindslev (113) has reported experiments in which animals immunized with an avirulent tubercle bacillus were desensitized by injections of tuberculin and then reinfected with virulent bacilli, the desensitizing injections being continued throughout the experiment. The desensitized animals are reported to have developed in most cases more tuberculosis than hypersensitive ones. In some cases, however, they showed less tuberculosis. Only 5 desensitized and 5 hypersensitive animals that were similarly treated and studied were allowed to survive as long as a month, and 4 of these animals were complicated by pregnancy. There are various other complications in the experiments which cannot be discussed here. It will suffice to state that the animals were infected

not by the injection of a measured amount of tubercle bacilli, but by exposing them individually for various periods of time to a spray of bacilli. It is obviously impossible in such a manner to ensure equality of the inhaled infecting dose. Indeed, in the only experiment in which the lungs of some of the animals were searched for tubercle bacilli during the first 24 hours after the inhalation, bacilli were found in the lungs of the desensitized animals but not in the hypersensitive ones. Even the most ardent advocate of hypersensitivity as a mechanism of immunity would hardly claim that tissues infected with tubercle bacilli can be sterilized within a few hours; and, as a matter of fact, other hypersensitive animals of the same experiment that were allowed to live developed tuberculosis.

Recently, Willis and his co-workers (95) (96) confirmed the demonstration that during a period of two months following a test infection immunized animals that are kept desensitized show as high a degree of resistance to tuberculosis as do immunized hypersensitive animals; but they stated that if the desensitization were continued for three to six months the animals showed more extensive lesions than hypersensitive ones, and this they suggested might be due to the lack of hypersensitivity. They noted, however, that the daily injections of the large doses of tuberculin caused the animals to develop large cutaneous ulcers and to lose their appetite and become markedly emaciated (96), and they suggested that "the inanition, emaciation and ulcers of the skin all might be factors in bringing about the difference" between the amount of tuberculosis in the desensitized and the untreated animals. That resistance to tuberculosis may decline after several months under such conditions is hardly surprising without drawing hypersensitivity into the question. Indeed, to anyone who has kept guinea pigs in a desensitized state by daily injections of large doses of tuberculin it is a matter of wonder that the treated animals retain their immunity during even two months of that drastic treatment. Regardless of what may have been the cause of the final depression of immunity after some months of such debilitating treatment, all investigators have agreed that, following a standardized infection, at the time when the non-immunized controls show widespread, extensive lesions throughout the body the immunized, desensitized animals have as few and as slight lesions as have immunized, hypersensitive ones. It is therefore hardly possible to accept the view that the inhibition of proliferation and spread of tubercle bacilli in the immunized body is due to the occurrence of a rapid hypersensitive inflammation at the site where the bacilli lodge.

Willis and his co-workers (95) also reported that if normal animals were infected with tubercle bacilli and were prevented from becoming hypersensitive by the injection of large amounts of tuberculin daily from the beginning of the infection, the animals so treated showed more extensive tuberculosis at autopsy than did animals that were allowed to become hypersensitive following infection. In a subsequent study of the same type (133) in which varying amounts of tuberculin were injected, there were 21 desensitized animals which did not react definitely to 1 and 2 mgm. of tuberculin (the only test dosages to which all groups were subjected) at any test during the course of the infection. Of these, 13 had more tubercle bacilli in their lungs at death than had hypersensitive controls; but 8 of them had no more bacilli (3 had even fewer) in their lungs than had animals that were hypersensitive throughout the entire period of the experiment. Willis and Woodruff concluded that these results "point to the existence of at least a partial reciprocal relationship between the allergic state of infected guinea pigs and the number of tubercle bacilli demonstrable in their lungs." In these studies animals that had received daily subcutaneous injections of 2 cc. of undiluted tuberculin showed more tuberculosis at autopsy than those receiving only fractions of a cubic centimeter of diluted tuberculin. In none of the studies of Willis and Woodruff, however, were there any control animals that were subjected to the trauma and debilitating influences incident to the daily subcutaneous injections of 2 cc. of undiluted, glycerin-containing tuberculin. Follis (27) has repeated their experiments, adding animals that were subjected to daily injections of control solutions in comparable amounts. He, too, found that some of the tuberculin-treated animals in which hypersensitivity was prevented from appearing developed more severe tuberculosis than did the untreated hypersensitive groups; but some of those injected with the control solutions developed altogether as severe lesions, even though they possessed hypersensitivity. Since two-thirds of the animals without hypersensitivity showed no more tuberculosis than did the untreated, hypersensitive ones, and since none of the desensitized animals showed more extensive lesions than were found among the hypersensitive ones that were treated with control solutions, it seems evident that the sporadic differences in the degree of tuberculosis observed in animals injected daily from the beginning of infection are due to some factor other than the presence or absence of hypersensitivity. It was noted by Follis (27) that, as in the case of injections of tuberculin, daily injections of the control solutions likewise caused loss of appetite and emaciation. Rothschild and his co-workers (26), also, have reported

the debilitating effect of daily injections of glycerine-containing solutions which did not depress hypersensitivity.

Birkhaug (141), in a very recent study of the same type as that of Willis and Woodruff referred to above (133), found that animals that were prevented from becoming hypersensitive (skin tests always with 10 mgm. of tuberculin) during the entire period of 5 months of infection with virulent bacilli, showed less tuberculosis than controls that were allowed to become hypersensitive.

Cummings and Delahant (79), Boquet (89) and Higginbotham (22) have also interfered with the appearance of hypersensitivity by administering tuberculin from the beginning of the infection in previously normal animals. In the first two studies hypersensitivity was greatly depressed, but not abolished; in the latter, it was completely prevented from appearing. The desensitized animals in these studies showed no less resistance to the infection than did controls that became hypersensitive following infection.

Balteanu, Toma and Garaguli (138), in order to prevent the appearance of hypersensitivity, gave daily desensitizing injections of tuberculin from the beginning of immunization with attenuated bacilli, and continued the injections throughout the course (5 months) of the test infection with virulent bacilli. The desensitized animals developed less tuberculosis than did immunized hypersensitive controls. Saenz (136) and Corper (144) carried out experiments of the same type, and they, too, found that the desensitizing treatment did not interfere with the development or maintenance of acquired resistance.

It is of particular interest that Willis (97), himself, shortly after his desensitizing experiments mentioned above, carried out a study of the effect of desensitization in human tuberculosis. The results of that study agreed with those of numerous other studies on the desensitization of tuberculous patients (135) (140) (152) in that the desensitization did not depress immunity, but was beneficial. Indeed, Willis wrote: "We were agreeably surprised at the improvement experienced by more than half of the fifteen persons treated" (97).

Topley and Wilson (98), in their discussion of hypersensitivity and immunity, have objected to the conclusion that the persistence of immunity after the abolition of hypersensitivity by tuberculin desensitization demonstrates the independence of immunity and hypersensitivity, and they base their objection solely upon the ground that "the possible immunizing effect of the tuberculin itself is altogether ignored." Several others have echoed this same curiously illogical objection. It

must be obvious that even if tuberculin did possess immunizing potentialities, that fact would in no way affect the demonstration, provided by the desensitizing experiments, that immunity can function effectively in the absence of hypersensitivity. But regardless of this, Topley and Wilson were apparently not aware of the fact that numerous fruitless attempts to immunize with tuberculin had already been recorded in the literature by competent investigators (114) (71) (119). Follis (106) has recently restudied the matter, and has shown that the treatment of normal animals with the same amount of tuberculin as in the above-mentioned desensitizing experiments, and over a comparable period of time, produces no immunity whatever. This conforms with the other recorded unsuccessful attempts to produce immunity by injections of tuberculin, and invalidates the objection of Topley and Wilson.

Wilson and his co-workers (137) have recently reported studies on desensitization in tuberculosis which confirm completely the results of the numerous investigators mentioned above who have shown that desensitization does not decrease immunity. Indeed, the desensitized animals in the studies of Wilson and his co-workers showed a greater resistance than hypersensitive controls. Four separate desensitizing experiments were carried out. The authors' conclusion regarding the first experiment is: "The results of this experiment strongly suggest that the effect of continuous desensitization on the animals in group B was to limit very considerably the progress and spread of the disease." Of experiment 2 they write: "On the whole the suggestion is that animals kept continuously desensitized after infection develop a less rapid and severe type of disease than normal animals." Of experiment 3 they write: "The results of this experiment are in general harmony with the conclusions drawn from the two previous experiments. They suggest that continuous desensitization after infection tends to limit the progress of experimental tuberculosis." Of experiment 4 they write: "The results of this experiment seem to show that animals kept more or less desensitized with O.T. have a significantly longer survival time and develop less severe tissue lesions than control animals." "These results," they continue, "though perhaps more definite, are in general consonance with those described by such workers as Rothschild, Friedenwald and Bernstein, Derick, Branch, and Crane, Branch and Kropp, Higginbotham, Follis, Balteanu, Toma and Garaguli, and Saenz." It will be recalled that the experiments of all of the latter workers showed that desensitization does not decrease immunity in

tuberculosis (26) (90) (92) (22) (27) (94) (136). Curiously, in spite of the above uniformly confirmatory results of their own experiments, and without adducing any contradictory experimental evidence, Wilson and his co-workers conclude by stating that they still prefer to hold to their belief that hypersensitivity is of value in increasing resistance to tuberculosis. They give as a reason for this belief their opinion that it is a "false assumption that desensitization is complete merely because the skin reaction to tuberculin is reported as negative," and that it is not proper "to conclude that the absence of a cutaneous reaction was proof of the concurrent absence of a systemic reaction." They, themselves, made no test of the systemic reaction in their experiments, and they were apparently not aware that it has been repeatedly demonstrated that proper desensitization abolishes systemic as well as cutaneous hypersensitivity. This was clearly shown in this laboratory by Rothschild and his co-workers (26), who demonstrated that desensitized animals do not react systemically to the intraperitoneal injection of amounts of tuberculin which regularly cause fatal systemic reactions in hypersensitive animals. This was confirmed by Siegl (88), who showed that desensitized animals tolerated without symptoms the intraperitoneal injection of amounts of tubercle bacilli which produced fatal systemic reactions in hypersensitive controls; and Branch and Kropp (92) showed that desensitized tuberculous animals tolerate without symptoms even the intravenous injection of the relatively enormous dose of 2 cc. of tuberculin, which invariably caused fatal systemic reactions in hypersensitive animals. In all of these studies the desensitized animals retained their immunity unimpaired. Not only do these facts invalidate the above-mentioned objection of Wilson, but it should be clearly remembered that the standard argument for the virtue of hypersensitivity has always been that it is the accelerated and accentuated hypersensitive *local* inflammation, occurring at the immediate site where bacteria lodge, that is responsible for the inhibition of their dissemination and for their more effective destruction in the immune body; and it has been amply proved that in desensitized animals hypersensitive inflammatory reactions do not occur where the specific bacteria lodge in the tissues (28) (26) (27) (22), and yet, in those and in the numerous other similar studies referred to above, the destruction of the bacteria and the inhibition of their dissemination (28) (136) (139) were as efficacious in the desensitized as in the hypersensitive body.

*The dissociation of immunity from hypersensitivity by spontaneous decline of sensitivity.* It is well known that hypersensitivity gradually

wanes after a tuberculous infection has become arrested (151). Does acquired resistance decline concomitantly? Numerous investigators (105) (71) have noted that the hypersensitivity of vaccinated animals gradually wanes with the passing of time, but that their acquired resistance to virulent infection remains intact. Willis (24) carefully re-investigated this question by immunizing animals with attenuated tubercle bacilli and testing their resistance by infecting them with virulent bacilli after the lapse of one to two years, at which time their hypersensitivity had declined to so low a level that they no longer reacted to the test doses of tuberculin. He found that in spite of the "greatly reduced and practically absent" hypersensitivity, acquired resistance to virulent infection remained intact. Willis, attempting to square this result with the view that acquired resistance is dependent upon hypersensitivity, suggested that the persistence of acquired resistance after "the virtual disappearance of the hypersensitive state" was due to the observed fact that following the virulent reinfection hypersensitivity reappeared in a decidedly shorter time than was required for non-immunized, control animals to become hypersensitive (*v.* the "amamnestic reaction" discussed in a previous section of this review). The present writer has elsewhere (72) set forth objections to that interpretation, and has pointed out that the experiments provide further evidence that acquired resistance is not dependent upon hypersensitivity. This viewpoint has recently been supported by interesting studies of Schwabacher and Wilson (145) and Wells and Brooke (146). These investigators, using much smaller numbers of bacilli in the test reinfection in experiments of the same type as those of Willis, found that the return of hypersensitivity was not accelerated, as it had been in Willis' animals, but, on the contrary, it was markedly delayed and suppressed in comparison with the rate of development in similarly infected but non-immunized controls. The acquired resistance of the immunized animals, nevertheless, remained intact in spite of the delayed development of hypersensitivity. These results render invalid the suggestion of Willis that the resistance of his animals to reinfection was due to an accelerated return of hypersensitivity. When very small numbers of bacilli are used in the reinfection, as in the experiments of Schwabacher and Wilson and of Wells and Brooke, the acquired resistance conferred by the previous immunizing infection inhibits the multiplication of the bacteria to such a degree that the production of a sufficient number of bacilli to effect sensitization can be markedly delayed, for the rapidity of sensitization depends largely

upon the number of infecting bacilli (147). These studies provide striking evidence that acquired resistance not only is independent of hypersensitivity, but that its operation can actually impede markedly the development of hypersensitivity.

*Hypersensitivity without immunity.* Since it is well established that acquired immunity can operate effectively in the absence of hypersensitivity, and since, as was pointed out above, a heterologous hypersensitive inflammation developing where bacteria lodge has no protective effect in the non-immune body (58) (59) (60) (61), attempts have been made to determine whether specific hypersensitivity to a given bacterium can exist in the absence of immunity to that bacterium. Seibert (107), Koch and Brudnicki (117), Smithburn, Sabin and Geiger (108) and Sabin and Joyner (5) showed that animals highly sensitized by injections of tuberculoprotein, whether alone (107) (108) or together with lipides (117) (5), had no immunity to infection with tubercle bacilli. From this result it was either implied or stated that true bacterial hypersensitivity had been established without concomitant immunity. However, the animals so sensitized differed from animals with true bacterial hypersensitivity in that they did not react hypersensitively to the local injection of tubercle bacilli or Old Tuberculin (5) (110); no mention is made as to whether the local reactions to the protein were immediate or delayed; no test of passive transfer of sensitivity was made in the latter three studies, and in Seibert's (112), the sensitivity was shown to be passively transferable. Boquet, Sandor and Schaefer (41) in similar studies showed that the test reactions in animals sensitized by injections of tuberculoprotein were of the Arthus (anaphylactic) rather than the bacterial type. In view of these facts, the sensitivity produced in the above studies must be regarded as having conformed to the Arthus rather than to the bacterial type; and since the animals did not react hypersensitively to tubercle bacilli, the experiments throw no light on the question whether specific hypersensitivity to a bacterium can exist in the absence of immunity.

Mackenzie and Woo (111) and Julianelle (118) have reported that animals sensitized to pneumococcal protein by repeated injections of the protein possessed no acquired resistance to pneumococcal infection. While the description of the sensitivity to the protein in the study of Mackenzie and Woo indicated that it may have been of the bacterial type, Julianelle (42) in an extensive study of the matter was never able to produce true bacterial hypersensitivity by injecting pneumococcal protein. As in the case of tuberculoprotein, the sensitization so pro-

duced always had the characteristics of Arthus sensitivity. Unfortunately, in the study of Mackenzie and Woo there was no test of the ability of the sensitized animals to react hypersensitively to the local injection of pneumococci.

Up to the present, therefore, no one has satisfactorily demonstrated that the injection of soluble bacterial antigens will produce bacterial type hypersensitivity to the specific bacterium in the absence of immunity. Indeed, as was stated earlier in this review, there is no acceptable evidence that the antigen responsible for the establishment of bacterial hypersensitivity has yet been isolated from any microörganism.

In a filterable virus infection McKee (143) observed the appearance of hypersensitivity to living virus without immunity, shortly after vaccination with killed virus. In such animals the progress of the infection was more rapid than in normal controls.

Weissfeiler (128) has reported that animals that were sensitized by the injection of a nonpathogenic acid-fast bacillus responded with marked hypersensitive reactions to the subsequent injection of virulent tubercle bacilli, but they exhibited no acquired immunity to the infection. Boquet and Nègre (70) obtained hypersensitive reactions at the site of injection of tubercle bacilli in animals sensitized by nonpathogenic acid-fast bacilli, but the immunity of the sensitized animals was not tested. Further studies on the establishment of hypersensitivity without immunity are highly desirable.

Résumé. The characteristic form of hypersensitivity that develops as a result of infection greatly increases the susceptibility of the body to injury by the proteins of the infecting microörganisms. As a result of this increased susceptibility of the tissues to damage, inflammation is more intense at sites where the microörganisms or their proteins lodge, and severe and even fatal systemic reactions may occur when the microörganisms, or even their bland proteins, enter the blood stream of the sensitized body. Because of these effects, the hypersensitive state is a factor that has seriously to be reckoned with in the interpretation of the lesions and symptoms of infectious diseases.

Bacterial hypersensitivity differs in a variety of ways from the anaphylactic and Arthus types of sensitivity. In the latter types, both sensitizing antigen and sensitizing antibody are readily obtainable, whereas in bacterial hypersensitivity there is no acceptable evidence that the sensitizing antigen has been isolated from any bacterium, and sensitizing antibody has never been satisfactorily demonstrated in the tissues or blood of the sensitized body. Nevertheless, there are rather

persuasive reasons for believing that bacterial hypersensitivity is dependent upon antibody.

While inflammation is an exceedingly important protective mechanism, it does not follow that in the body with acquired immunity an exaggeration of the inflammatory process by means of hypersensitivity is either necessary for protection or desirable; and this particularly since the hypersensitive tendency to accentuated inflammation is inseparably bound up with an increased susceptibility of the body to injury by the bacterial products. It must also be remembered that it has been clearly shown that in the hypersensitive body not only the fixed tissue cells, but also the protective phagocytes are far more susceptible to the damaging effects of the bacterial products than are the phagocytes of the non-hypersensitive body (6) (7) (8). The study of the tissues in the above described experiments in which immunity was dissociated from hypersensitivity has shown that acquired immunity enhances so greatly the protective power of the inflammatory exudate that, instead of requiring an exaggeration of the inflammatory process, an astonishingly small amount of inflammation (far less than occurs in the non-immune body as a result of the injection of the same number of bacteria) suffices to restrain the bacteria effectively. In this, acquired immunity parallels native immunity. The body that is natively immune to a given bacterium does not respond to that bacterium with an exaggerated inflammation and destruction of tissue. On the contrary, even in the instances in which the phagocytes are essential for the destruction of the bacteria, it ordinarily requires only a minute amount of inflammation to sterilize the tissues, and the tissue damage is negligible. Studies by Rich and McKee, in which the development of inflammatory exudate was markedly suppressed by treatment with benzol, have shown in a striking manner how slight an amount of exudate suffices to sterilize the tissues in both native (116) and acquired (66) immunity.

In relation to native immunity, it is pertinent to the matter before us that there is no parallelism between the degree of native resistance and the rapidity and degree of development of hypersensitivity following infection. If hypersensitivity were a potent mechanism of protection it might reasonably be expected that the species which developed it most rapidly and in high degree would be the best protected. This, however, does not prove to be the case. For example, both the guinea pig and the white rat are susceptible to the tubercle bacillus, but the progress of the infection to a fatal termination is far more rapid in the

guinea pig, which quickly develops a high degree of hypersensitivity following infection, than it is in the rat, which does not develop tissue hypersensitivity during the entire course of the infection (120). Furthermore, Lewis and Loomis (125), in a study of the reactions of inbred strains of guinea pigs with different degrees of native resistance to tuberculosis, found that the strain which exhibited the most marked hypersensitive reactions following infection was the least resistant to the infection. Lurie (126), from a similar study of inbred families of rabbits, states that "the acquired responsiveness of the tissues to tuberculin is not definitely related to resistance." A parallel to these studies exists in the human being. Tho writer (148) has presented evidence, supporting the studies of other investigators, which indicates clearly that negroes have a lower degree of native resistance and are less able to develop and maintain acquired resistance to tuberculosis than whites. Their capacity for developing hypersensitivity is, however, greater than that of whites. Cummins (150) has stressed the markedly higher degree of hypersensitivity and the decidedly lower degree of resistance of the South African native as compared with European whites; and in this country Pinner and Kasper (149), from a careful study of the matter, state that their results "lead necessarily to the conclusion that infection tends to produce a high state of allergy in the American negro, but that he, unlike the white, does not readily acquire a state of increased resistance coincidently."

One of the standard arguments that immunity in tuberculosis depends upon hypersensitivity has been the fact that the antibodies present in the serum of the immunized body have not been satisfactorily shown to protect normal animals against infection. The demonstration of the effectiveness of antibodies by passive transfer in a chronic infection in which, even in the actively immunized body, the immunity is imperfect and the destruction of the microörganisms is slow and difficult, is much less readily accomplished than in the case of acute infections in which acquired immunity is more effective. For years syphilis, malaria and tuberculosis have been regarded as infections in which immunity must be "cellular," and the antibodies of no protective value. Recently, however, in both syphilis and malaria the serum of the immunized body has been demonstrated to possess the power of passively protecting normal animals (115) (122). It is reasonable to expect that in tuberculosis, likewise, proper methods may yet reveal the protective rôle of the antibodies that appear in the serum of the immunized body.

Willis and Woodruff (133), pleading the cause of hypersensitivity as a

mechanism of immunity in tuberculosis, compare this infection with smallpox, stating that in both of these infections "immunity resides in the cells," and that, in both, hypersensitivity and immunity coexist. In the case of tuberculosis the statement that immunity is "cellular" can be only a supposition, in view of the present lack of understanding of the significance of the antibodies that are present in the serum of the immunized body. In smallpox the immunity certainly does not reside exclusively in the cells, for protective antibodies, demonstrable by passive transfer, are present in the serum. But regardless of this, smallpox provides a particularly interesting example of an infection in which the available evidence speaks against, rather than in favor, of the necessity of hypersensitivity for the operation of immunity. Rivers, Haagen and Muckenfuss (134) have shown that the cells of the immunized body are resistant to vaccinia virus even *in vitro* in tissue culture, in the absence of inflammatory cells and of plasma of the immune body. There is, therefore, no reason to believe that the hypersensitive inflammation which appears at a site of infection in the intact body is in any way essential for the protection of the cells against the virus.

Many who have held the view that hypersensitivity is an essential or important mechanism of immunity have stated that one of the most persuasive reasons for that belief has been the probability that a phenomenon of bodily reactivity that is common to so many infections must serve a beneficial purpose. It may be recalled, however, that hypersensitivity to all sorts of non-bacterial proteins, resulting in conditions such as asthma, hay fever and eczema, is exceedingly common, but in no quarter are those hypersensitive reactions regarded as essential protective devices. On the contrary, every effort is made to abolish the hypersensitivity by desensitization, and the results are highly beneficial.

The studies described in this review have shown, by a variety of methods and in widely different types of infection, that neither of the two basic manifestations of acquired immunity (the inhibition of spread of bacteria and the suppression of their proliferation) are dependent upon hypersensitive inflammation. The accumulated evidence has caused a revision of the generalization that hypersensitivity is necessary for the successful operation of acquired immunity, that hypersensitive destruction of tissue is a price that must be paid for the protective effects of acquired immunity, and that tests for hypersensitivity can serve as tests for the level of acquired immunity. It is, of course, possible that

under certain as yet unknown conditions at certain periods of certain infections, hypersensitive inflammation may be necessary for protection; and in our ignorance of the true significance of all forms of hypersensitivity every effort should be directed toward the discovery of such conditions, if they exist. It is, however, of no value merely to reiterate the unproved assumption that hypersensitivity is "a stage in the development of immunity" (98). It is fair to say that, up to the present, hypersensitive inflammation has never been satisfactorily shown to be necessary for the successful operation of acquired immunity at any stage of any infection under any condition whatsoever. On the other hand, in a wide variety of infections acquired immunity has repeatedly been shown to remain intact when the capacity to react with hypersensitive inflammation has been eliminated by appropriate procedures. Since this has been demonstrated, some writers have expressed the opinion that even though the view that acquired immunity depends upon hypersensitive inflammation has not stood the test of careful examination, nevertheless hypersensitivity may embody some undefined protective mechanism which may persist when the recognized inflammatory-necrotic element has been eliminated (32). That is a viewpoint which cannot profitably be discussed until the hypothetical protective device has been defined, and has been demonstrated to be a part of hypersensitivity.

When numerous thoughtful students of a subject entertain divergent views about it, the extreme of each view is usually wrong. In the present instance one should certainly avoid both the view that hypersensitivity is always deleterious and the view that it is always essential for protection. Hypersensitivity may be regarded at present as a condition which in some instances is decidedly deleterious, in some instances exists without exerting any appreciable deleterious or beneficial effect, and in some instances may possibly serve as a useful auxiliary to the other forces of immunity. That hypersensitivity can exert deleterious effects has long been clear to everyone. That it is not essential for the successful operation of acquired immunity under a wide variety of tested conditions has now been demonstrated unequivocally. That under some, as yet undetermined, conditions acquired immunity may require the participation of hypersensitivity for its most effective operation is possible, but this possibility has not yet been established in the case of any infection. Dogmatic generalizations have long obscured unsolved problems relating to hypersensitivity. Because of its important influence upon the body during infectious diseases it is highly

desirable that efforts be continued to determine whether there exist any conditions at all under which the presence of hypersensitivity should be encouraged, and to define more precisely the conditions under which the abolition of the hypersensitive state by desensitization can be expected to be beneficial to the infected body.

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# PHAGOCYTOSIS OF FOREIGN MATERIAL IN THE LUNG[1]

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The scope of the present review will be confined to a discussion of the manner in which foreign material enters the lung, its distribution within the different regions of the airways and lung parenchyma and the mechanism of disposal of such material with especial consideration of phagocytosis by the several types of cells taking part in this process. The general subject of phagocytosis, including the mechanism by which certain cells engulf foreign particles, has been so ably discussed by Mudd, McCutcheon and Lucké in a previous review (1) that an account of these more fundamental aspects of phagocytosis can well be omitted in this communication. Since the literature on the subject is concerned principally with mammals it will be assumed that the observations recorded refer to this class of vertebrates except where specifically mentioned. The various kinds of foreign particulate matter, non-living (irritating and non-irritating) and living (pathogenic and non-pathogenic) which enter the lung will be dealt with separately.

Limitations of space do not permit a detailed description of the finer anatomy of the lung. For complete accounts of this subject the reader is referred to the extensive studies of Miller (2) and more recently those of Bargmann (3), Seemann (4), Joselyn (5), Loosli (6) and Macklin (7). The nomenclature proposed by Miller for the several divisions of the bronchial tree will be employed in this discussion, namely, in the order of finer divisions, bronchiolus, respiratory bronchiole, ductus alveolaris leading into the alveolar atria into which the groups of alveoli and individual alveoli open. The ductus alveolaris and its ultimate divisions is considered as an anatomical respiratory unit or primary lobule. The continuous epithelial lining ends with the ductus alveolaris. Ciliated epithelial cells extend only to the first part of the respiratory bronchiole. The wall of the alveolus is composed of an interlacing network of capillaries supported by a framework of elastic and collagenous fibrils and contains varying numbers of mononuclear, "septal" cells. These cells include the so-called "alveolar epithelium," histiocytes such as are found in the connective tissues, as well as undifferen-

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tiated mesenchymal cells and fibroblasts. The capillary walls are in direct contact with the air space except for a thin ground membrane and occasional septal cells attached to the wall which do not form a continuous layer. Between the capillary loops are frequent window-like openings (pores of Kohn) which constitute direct communication between adjacent alveoli (6, 7). The plexus of lymphatics surrounding the bronchi does not extend beyond the ductus alveolaris. The only vessels in the alveolar walls are capillaries.

ENTRANCE OF FOREIGN MATERIAL INTO THE LUNG. Since the lung occupies a unique position among the internal organs of the body in that it is more constantly in contact with the external environment than any other structure within the body cavity, it became essential early in the evolution of air breathing animals that an effective means for the exclusion and elimination of noxious material from the lungs be developed. The sinuous course and the sticky mucoid surface of the upper air passages represent the initial barrier against entrance of particulate air-borne material into the lower respiratory tract. Particles penetrating this barrier come in contact with the mucus covered surface of the trachea and bronchi where they tend to adhere and, depending on the size and nature of the particle and the time element, may be largely or completely prevented from reaching the alveoli. The experiments of Barclay, Franklin and MacBeth (8) are particularly informative in this respect. These authors insufflated into the trachea of cats, bismuth carbonate dust of a particulate size 3–12 $\mu$ and determined the distribution of this substance in the lung by means of an x-ray technique which they assert is capable of detecting particles as small as 0.15 mm. They found that this material was confined entirely to the bronchial tree. None was observed to reach the alveoli although the size of the particles was such that they could easily have passed through the ducti alveolari.

That particulate matter in dry form does penetrate to the alveoli is evident from many observations both clinical and experimental. The time of exposure to air containing such material appears to be one of the principal factors in this respect (9, 10, 11). It is well known that the amount of carbon in the lungs of city dwellers increases with age. Particle size (12, 10), concentration in the air (13, 14) and depth of respiration (15) are other important determinants. Brown (16) believes that dust is retained in the lung in proportion to its wetability. The work of Jones (17) on cultures of the lungs of normal animals is especially informative. He found that bacteria of various kinds could

be isolated from the periphery of the lungs of laboratory and domestic animals whose diet consisted of hay, oats and other dusty substances. When the food was subjected to preliminary washing or changed to non-dusty material the number of bacteria found in the lungs was much diminished. In harmony with the rôle played by dust in this respect is the finding that the lungs of certain carnivors, e.g. the dog, are for the most part sterile (18) as are the lungs of human beings (19).

Many fewer data are available on the capacity of inspired fluid droplets to reach the alveoli and deal almost entirely with the inhalation of finely divided suspensions of bacterial cultures. Stillman and Branch (20) exposed rabbits and mice to such atmospheres containing streptococci, pneumococci, staphylococci and other bacteria and at short intervals thereafter were able to isolate these microörganisms from the periphery of the animal's lungs. Wherry and Butterfield (21) performed similar experiments earlier with B. enteritidis and pneumococci and were able to isolate only pneumococci from the peripheral lung tissue. In unpublished experiments Hamburger and the author (18) sprayed cultures of pneumococci into the lungs of dogs and within five minutes after completion of the procedure obtained pneumococci in pure culture from snips of tissue taken from the surface of the lung.

Whether minute droplets penetrate to the alveoli more readily than equally fine dry particles we do not know. Such data would be of great value in contributing to a better understanding of the inception of certain pulmonary infections. The nearest approach to information on this subject is provided by the work of Trillat (22) who found that exposure of small laboratory animals to very finely divided droplets of several bacterial species resulted in penetration of the bacteria to the depths of the lungs in a very short space of time. Whereas if such bacteria were mixed with talc and the animals allowed to breathe this dust, microörganisms did not reach the terminal airways. The use of a simple bacterial dust would have been much more informative since the size of the talc particles may have been such as to prevent deep penetration of the bronchial tree.

Fluid entering the lower respiratory tract is distributed in a manner quite different from that of air borne material. Such liquid material passing the barrier of the epiglottis tends to flow directly to the terminal parts of the lung, depending on the viscosity of the fluid, the position of the body and other factors less well understood (23, 24). Barclay's experiments (8) bring out strikingly the difference in distribution between air-suspended and fluid-suspended radio-opaque substances.

While the former were confined to the bronchi, the latter penetrated quickly into the alveoli. The fact that the epiglottis constitutes by no means a perfect barrier to the passage of material from the upper to the lower respiratory tracts has been appreciated only in recent years. Clinicians have long felt that the pathological condition of broncbiectasis was due principally to the implantation within the bronchi of infected exudate from the locally diseased areas in the nose and throat. But it was not until the relationship between lipoid pneumonia and oily nose drops was recognized by Laughlin (25) that this subject received special investigation. The experiments of Walsb and Cannon (26, 27) demonstrated clearly that liquids dropped into the nasal passages of animals quickly find their way into the depths of the lungs and if irritating in nature produce an inflammatory reaction in the alveoli and lung parenchyma. Webster and Clow (28) were able to produce pneumonia in mice by the nasal instillation of relatively large quantities of pneumococcus culture.

Conditions which cause failure of the epiglottis to close completely are not well understood but an important contribution to our knowledge on this subject has recently been made by Nungester and Klepser (29). These workers, by means of an ingenious technique, measured the closure of the epiglottis in rats and found that lowering the surface temperaturo of the animal resulted in incomplete closure. Under such conditions they found that India ink mixed with mucin and placed in the nares found its way into the lungs in considerable quantities in more than half the test (chilled) animals, while in the normal nonchilled controls very little or no ink was detected in the lungs. Using the same technique they were able to induce pneumonia in chilled rats by the intranasal instillation of pneumococci suspended in mucin in 42 per cent of the rats while infection occurred in only 13 per cent of the nonchilled controls similarly injected.

Elimination of foreign material from the lung. The mechanism for the removal of foreign material which has not penetrated the lung beyond the terminal bronchi is under ordinary conditions very effective. This consists principally of the ciliary movement, which is capable of sweeping along particles at a surprising rate, 0.25 to 1 cm. per minute in the bronchi (30) and up to 3 cm. in the trachea (31).[2] The expulsive action resulting from the narrowing and widening of the

[2] In a series of beautifully executed experiments Proetz (32) has contributed important information on the conditions under which the functional activity of the nasal ciliated epithelium is disturbed or inhibited.

diameter of the bronchi with each respiration, the possible peristaltic movement of the bronchi (33, 34)[3] and cough all aid in the eliminatory process by way of the trachea. Extraneous material entering the alveolar units is disposed of much less easily. The principal means employed by the body for the immobilization and removal of such matter is that of phagocytosis by certain large amoeboid cells, the alveolar phagocytes or "dust cells" of Von Inns, normally present in small numbers in the alveolar spaces and mobilizable in large numbers in response to certain types of stimulation. Following engulfment of particulate material many of these cells migrate from the alveoli into the ductus alveolaris—some are then eliminated through the air passages, others pass into the lymphatic system and are deposited into the peribronchial and perivascular lymphoid nodules in the lung or carried to the hilum lymph nodes (35, 36, 37, 38). The termination of the ductus alveolaris appears to be the principal locus of entrance into the lymph channels of the bronchi and blood vessels. Certain kinds of minute particles may pass directly into the lymphatics without the intermediate step of phagocytosis. Drinker and Field (39) conclude from their experiments that this is the case with suspensions of silicate of nickel, manganese and fat particles. Hamburger and the author (18) found pneumococci in the hilum lymph nodes but not in the blood within ten minutes after spraying cultures of pneumococci into the lungs of dogs. While the usual pathway of pathogenic bacteria from the terminal air sacs to the blood is most likely by way of the lymphatics to the hilum lymph nodes, they may under special instances take a shorter route. Tuttle and Cannon (40) found that virulent hemolytic streptococci can penetrate directly from the lung of the dog into the blood stream without apparently passing through the hilum lymph nodes. There seems to be no unquestionable evidence that particulate matter, either living or nonliving, reaches the lymphatics by passage through the intact bronchial wall in regions proximal to the respiratory bronchiole (27, 41, 11).

Cells participating in phagocytosis. *Type of Cell.* The kind and degree of cellular reaction elicited by the presence of foreign particulate matter in the terminal airways of the lung seems to depend principally on the nature of the material. Highly irritating materials,

[3] Jarre (33) has presented good radiographic evidence for the occurrence of a peristaltic wave during expiration which moves from the more distal portions of the bronchi toward the hilum. Such peristaltic waves were not observed in severe bronchial infection. However, Gordonoff (34) was not able to confirm Jarre's findings.

living as well as non-living, produce a local exudation of blood cells, principally polymorphonuclear leucocytes together with considerable numbers of red blood cells and plasma (edema fluid). This type of cellular response characterizes the initial inflammatory reaction in the acute bacterial infections of the lung and in certain of the more chronic ones, e.g., tuberculosis. Likewise, such substances as starch and aleuronat (suspended in nutrient broth) injected into the lung call forth the same kind of cellular reaction. On the other hand, inert and relatively non-irritating materials, dusts, carbon, oil droplets, particulate dyestuffs, silica and others cause a much slower inflammatory reaction which is characterized by the mobilization of mononuclear cells, principally macrophages. Both types of cells, the polymorphonuclear leucocyte and the macrophage, are actively phagocytic but differ in their ability to engulf and digest various kinds of foreign particles. Such differences will be discussed in detail in the subsequent sections as well as the rôle played by the monocytes, bronchial epithelial cells and endothelial cells of the pulmonary capillaries.

*The Macrophage.* a. *Origin.* The problem of the origin of the macrophage is one which has concerned cytologists for half a century or more. It is quite outside the scope of this paper to undertake even a brief consideration of the differing ideas concerning their derivation. These concepts have been graphically stated by Gay (42). It will suffice here to present the most generally accepted views. The early work of Maximow (43), Evans (44), Aschoff (45) and others showed that these cells and their parent forms were widely distributed throughout the body both as free cells, found principally in the spleen, bone marrow, and connective tissues as well as fixed cells lining the sinusoids of the spleen, bone marrow, lymph nodes and liver. The septa of the pulmonary alveoli constitute another potential source for macrophages (46). Those in the liver appear to be a specialized type in that aside from their larger size they are by far the most actively phagocytic of the fixed macrophages.

Concerning the origin of the macrophages from the lining cells of the sinusoids of the organs above mentioned and the widely distributed reticular tissues, there is little disagreement. But opinion has varied widely as to whether these cells also represent developmental forms of certain of the blood cells, namely, lymphocytes and monocytes. While numerous investigators, Lewis and Lewis (47), Clark and Clark (48), Simpson (49), Elliott (50), Forkner (51) and others believe that the monocytes may be and often are transformed into macrophages, these

authors do not feel that there is sufficient evidence to include the lymphocytes as an earlier stage in the evolution of the macrophages. However, other workers, notably Maximow (52), Bloom (53), Taliaferro and Cannon (54), and Loosli (55) have produced what seems to the writer as convincing proof as can be obtained, that under certain conditions lymphocytes may develop into macrophages through the stages of the monocyte or polyblast. Bloom has observed such developmental sequence in carefully studied tissue cultures of mammalian lymph. These concepts are at variance with the conclusions of Sabin and co-workers (56) that macrophages arise from endothelial cells and monocytes from reticular cells but many workers feel that it is not possible to differentiate the monocytes from the various morphological and physiological manifestations of the macrophage.

b. *Alveolar phagocytes—origin.* The origin of the alveolar phagocytes, both normally present and accumulating in response to local irritation, is also a subject of controversy. Disagreement on this subject is due in part to differing concepts of the anatomy of the alveolar wall. The persistence of the older and probably erroneous view that the air sacs are lined with a continuous layer of epithelium, co-extensive with the lining cells of the bronchi, has led many investigators, who believe in the local origin of these cells, to the conclusion that the alveolar macrophages represent a transformation of these epithelial cells into free moving phagocytes. Since such a metamorphosis is unknown elsewhere in the body, it would be necessary to hypothecate, as Aschoff (57) has done, a special type of epithelial cells for this region. The structure of the alveolar wall as described at the beginning of this discussion offers no particular difficulty in ascribing a local derivation for the alveolar phagocytes. Indeed, there is a growing body of evidence to indicate that the alveolar macrophages arise locally from preëxisting septal cells. Certain authors, Briscoe (58), Sewell (59), Carlton (60), and Cappell (61), conclude that these cells develop from what they designate as the alveolar epithelium. These lining cells hypertrophy, show a cytoplasmic change from a finely granular to a foamy character and detach themselves from the alveolar wall. Other workers, Lang (46), Gardner and Smith (62), Fried (63), Lemon and Higgins (64), Tuttle and Cannon (65), and Loosli (66), ascribe to them a more general derivation, namely, from the mononuclear cells of the septum and the interstitial tissues.

Studies by the author and associates (67, 68, 69) of the cellular changes occurring during the evolution of the lesion of lobar pneumonia, both in animals and man, afforded an unusual opportunity for observing the

gradual transformation of the septal cells into free macrophages. Within 24 to 36 hours after the inception of the experimental disease in the dog, large nuclei surrounded by a darkly staining cytoplasm began to appear in increasing numbers in the substance of the septum and on its surface. These cells enlarged and those on the surface exhibited at first a fine, and later a coarsely foamy or reticulated appearance most pronounced in the unattached part of the cell. Within 36 to 48 hours these cells began to separate from the alveolar wall and entered the exudate as actively phagocytic macrophages. Whether these macrophages arise chiefly from the preëxisting lining or exposed cells or from those within the septum was not determined. Nor was it possible to estimate how many of the macrophages in these exudates developed from the local tissue cells and how many from the blood lymphocytes and monocytes mobilized in the early inflammatory exudate (55). It has been noted by practically all observers that mitotic division of the proliferating septal cells is of infrequent occurrence, although under special conditions such as the early reaction to tubercle bacilli mitotic division of the mononuclear cells of the alveolar wall has been described (70). Free binucleate macrophages, on the other hand, are not uncommon, but whether or not this represents amitotic division is uncertain.

c. *Functional activity in relation to stage of maturation.* Concerning the stage in its development at which the macrophage exhibits phagocytic activity, there exists much diversity of opinion. This applies particularly to the septal cells on the walls of the alveoli. Certain investigators, beginning with Tchistovitsch in 1889 (71) have not observed phagocytosis of foreign particles by the cells attached to the alveolar wall. Various substances introduced into the terminal air spaces have been used to determine the activity of the attached cells. Haythorn (11) employed lamp black inhaled over considerable periods of time. Ross (72) used lipiodol. Gardner (35) observed a large number of animals during inhalation of various kinds of dusts. None of these authors detected phagocytosis by the fixed cells. In our study (73) of the injection of pneumococci into the resolving lung of canine pneumonia we rarely observed microörganisms within the attached macrophages, whereas great numbers were engulfed by the free ones. On the other hand, many workers have reported phagocytic activity by the septal cells; Briscoe (58), using pigeon erythrocytes in the guinea pig, Carlton (60) with inhaled coal dust, Cappell (61) with India ink and carmine, Fried (63, 74) with pyrrhol blue and oils, Lemon and Higgins (36) with silica, Tuttle and Cannon (65) with staphylococci

wise the upper limit of the size of mineral particles phagocytable by the macrophages (85, 86, 13). It may be assumed from these observations that larger particles do not usually reach the alveolar spaces.

The characteristic lesions found in the lungs of patients dying with silicosis have been produced in experimental animals by inhalation over long periods of time and by intratracheal injection of pure mineral dusts composed of silica, serecite, borosilicate glass, quartz and others. The most complete descriptions of this pathological process have been given by Gardner and co-workers (10, 35, 87, 88) and Lemon and Higgins (86, 89, 90). Gardner (10) found that it required a matter of many months' to years' inhalation of quartz dust before any significant changes in the lungs occurred. The mineral particles are however early engulfed by the macrophages which begin to accumulate in the alveoli and then move relatively rapidly into the lymphatics and lymphoid tissues where they cause marked proliferation of the fixed tissue (mononuclear) cells. The migration of silica-bearing macrophages is much more rapid than that of macrophages containing non-siliceous dusts. These latter often remain for long periods in the alveoli. When these silica containing materials are injected intratracheally, as observed by Lemon and Higgins (89), the whole process of phagocytosis, migration into the lymphatics and proliferation of fixed tissue macrophages was greatly accelerated, presumably because a larger amount of irritating material was deposited rapidly in the alveoli. Gardner noted relatively little reaction in the alveolar wall cells. Lemon and Higgins have described early desquamation of the alveolar epithelium and migration of phagocytic cells into the alveoli. The latter authors also observed phagocytosis of silica by the attached septal cells.

The macrophages retain the silica particles and may show little functional disability for relatively long periods of time. In some of their experiments Lemon and Higgins (86) found evidence of degeneration of macrophages containing borosilicate glass after 2 months. Other authors (91, 92) have observed no evident injury of serecite-containing macrophages after 3 to 6 months. However, most workers agree that many of the macrophages ultimately degenerate and liberate the engulfed silica which is then taken up by new cells. Before such degeneration, giant cells are frequently formed (92, 88). The cause of the formation of fibrous tissue which is a characteristic feature of the silicotic nodule is not altogether clear. It is believed by most observers that it is the result of irritation and injury due to the silica liberated into the interstitial tissues by the degenerating macrophages. In harmony

with this idea is the observation that non-siliceous dusts which cause no injury to the macrophages do not produce pulmonary fibrosis (88). Others believe that the obstructed lymph flow occasioned by the marked accumulation of silica laden macrophages in the lymph channels is sufficient cause for the proliferation of fibroblasts. Such fibrosis has been observed in pronounced anthracosis (11). Lungs injured by silica are abnormally susceptible to infection with the tubercle bacillus and Gardner (35) found a high incidence of epizoötic pneumonia among his experimental guinea pigs exposed for long periods to quartz dust. The polymorphonuclear leucocytes appear to take no part in the disposal of silica.

Asbestosis is another condition closely allied to silicosis but differs from the latter in that the particles of asbestos which contain one or more of the silicates are of such a size as to preclude their penetration beyond the respiratory bronchioles.[4] This results in a somewhat different type of cellular reaction. Macrophages accumulate around the asbestos fibres, which are too large to phagocyte, and form giant cells in which the so-called asbestos bodies appear. The nature of the asbestos body, a long club shaped rod consisting of an outer layer of protein material and an inner one of changed asbestos (93, 94) suggests that it is the resultant interaction of phagocytic cells and asbestos. Fibrosis of the surrounding interstitial tissues occurs much more slowly in this condition than in experimental silicosis. The non-siliceous earths are much less irritating and while they may cause marked accumulation and retention of macrophages in the alveoli, they do not cause fibrosis. Two papers by Gardner (95, 88) contain an excellent review of the etiology and pathology of pneumoconiosis as does also the volume on silicosis and asbestosis edited by Lanza (13).

Lipoids. One of the most striking and perhaps the most spectacular example of phagocytosis in the lung is that afforded by the reaction of the macrophages to the presence of oils and fats. Study of this subject has grown out of the relatively recently recognized condition, lipoid or lipid pneumonia. Since Laughlin's original description (25) there have been a number of excellent contributions both clinical and experimental by Pinkerton (96, 77), Fried (97), Cannon and Walsh (98), Patterson (99), and Graef (100), just to mention a few. This type of pneumonia has been caused by a variety of oils and fatty materials both mineral and animal in nature and include such substances as albolene, liquid

[4] Gardner (95) states that asbestos fibres 150–200μ long have been found in the alveoli.

on culture and showed on microscopic examination no evidence of inflammation. It was also found that transportation of the pneumococci by way of the lymphatics to the hilum lymph nodes played a rôle in this process of removal from the lung. Stillman (106) observed that while pneumococci inhaled by mice into the depths of the lung disappeared within a few hours from the lung tissue, hemolytic streptococci, staphylococci and B. Pfeiffer remained there for relatively long periods of time.

PHAGOCYTIC ACTIVITY OF THE NEUTROPHILIC LEUCOCYTES. When, however, pathogenic and, under unusual conditions, non-pathogenic bacteria are implanted in the air sacs in considerable numbers, an accessory mechanism is required to deal with the foreign irritating material. This consists of an outpouring of fluid and cells from the blood into the alveolar spaces. The initial cellular response to all the common types of bacterial pulmonary invasion, pneumococcus, streptococcus, staphylococcus,[6] B. Pfeiffer, B. Friedlander, and most of the uncommon ones, meningococcus, gonococcus, B. typhosus, etc., is predominantly neutrophilic in character. The polymorphonuclear leucocytes are actively phagocytic and engulf the microörganisms in varying numbers depending on the opsonic properties of the blood plasma. In fact, the phagocytic activity of these cells for pathogenic bacteria has been shown to be conditioned entirely by the concentration of opsonins for the specific invader. The leucocytes of animals, whose blood serum possesses no detectable opsonic action for a given bacterium, show little or no ability to phagocyte this microörganism no matter how much of the animal's serum is present, whereas these same leucocytes, when mixed with the same bacterium which has been previously exposed to the serum of an animal possessing marked opsonic properties, exhibit active phagocytosis (107).

The ability of the polymorphonuclear leucocytes to engulf pathogenic bacteria constitutes the principal factor in the so-called localizing power of the lung, i.e., a retardation of the growth of the microörganisms and of spread to adjacent tissues including the blood stream.[7] If this pro-

[6] The finding of Tuttle and Cannon (65) that staphylococci injected into the lung of a dog were phagocyted practically entirely by the septal cells may represent a special instance of the body's reaction to a microörganism of relatively low virulence for the animal species.

[7] Another localizing factor has been described by Cannon and Pacheco (108) and Rich (109), namely, agglutination and adhesion of the microörganisms to the tissue cells.

tective mechanism is defective or lacking, the animal may succumb to the infection before the body can bring other defense reactions into play. The degree to which the lung can localize implanted bacteria depends on a number of variables; the size of the infecting dose of microorganisms, the rate of mobilization of leucocytes at the site of infection, the concentration of opsonins in the blood plasma, the virulence of the microörganisms, the body temperature and other factors less well understood or not known. If the localizing power is high the infection may be terminated very quickly. If present to a lesser degree the inflammatory lesion extends but at a relatively slow rate.

The neutrophilic leucocytes possess proteolytic enzymes which are capable of digesting most kinds of engulfed bacteria. Some microorganisms, such as the gonococcus, appear to remain in the cells with little evidence of digestion. But whether such bacteria are capable of multiplication is not known. That certain kinds of bacteria are rapidly injured after ingestion even though they may appear morphologically intact has been shown by Gregg (110) with the pneumococcus. The digestive activity of the leucocytes appears to diminish much with the age of the cell (80) and the numbers of phagocyted microörganisms. We have observed that neutrophiles which have engulfed very large numbers of pneumococci are unable to digest them. Furthermore, the leucocytes may be so injured by the toxic products of the microorganisms in the surrounding medium that they lose their functional activity for either phagocytosis or digestion or both and engulfed living bacteria may be liberated (111).

The rôle played by the neutrophilic leucocytes in the body's reaction to pneumococcus infection has been studied in detail by the author and associates (112) in experimental canine pneumonia, and by Gunn and Nungester (113) in the rat. These cells accumulate early at the site of the implanted microörganisms and within a few hours begin to phagocyte the pneumococci. As the intensity of the cellular mobilization increases, more and more of the bacteria are engulfed and in the central parts of the lesion they are seen to be in the process of digestion within the leucocytes. Meanwhile many pneumococci are present in the spreading margins of the inflammatory process where they lie free in the edema fluid, such exudation being a characteristic response to the growth of pneumococci in the body. Extension of the lesion occurs as this infected edema reaches adjacent tissue by way of the smaller air passages and the interalveolar pores of Kohn. Were it not for the retarding effect of phagocytosis the lesion would spread with great

rapidity throughout the lung as indeed is observed to be the case when large doses are employed. In such lesions little phagocytosis is observed.[8]

RÔLE OF THE MACROPHAGE IN PNEUMOCOCCUS PNEUMONIA. However, despite the phagocytic activity of the leucocytes, the lesion in lobar pneumonia usually extends to involve a whole lobe and may spread to other lobes of the lung even in the animal that is destined to recover. It is evident that in order to bring about recovery from the infection the body must either increase the so-called natural means of antibacterial defense described above or bring into play a new mechanism. There is no evidence for a change in the former defense process but quite clear evidence that a new type of reaction occurs at the onset of recovery by which the pneumococci are rapidly destroyed and the disease terminated. This consists in the occurrence in the body fluids of much more potent opsonic properties and the appearance in the pneumonic exudate of increasing numbers of macrophages which arise both from the fixed tissue cells of the lung parenchyma (67) and the mononuclear cells of the blood (55).[9] In such lesions the macrophages were seen to be actively engulfing and destroying the pneumococci whereas the polymorphonuclear leucocytes at this stage of the disease, while usually still exhibiting phagocytic activity, appeared to have lost their ability to digest the intracellular pneumococci.

The same sequence of changes was observed to occur in both human and canine pneumococcic pneumonia (68) except that the evolution of the inflammatory process is much slower in the human being.

RELATIVE PHAGOCYTIC AND DIGESTIVE ACTIVITIES OF MACROPHAGES AND NEUTROPHILIC LEUCOCYTES FOR PNEUMOCOCCI. The foregoing findings gave rise to numerous questions concerning the relative phagocytic and digestive activity of the macrophages and neutrophilic leucocytes for pneumococci. The author and Van Sant (80) undertook a study of this subject employing macrophages and polymorphonuclear leucocytes secured from gum arabic and aleuronat pleural exudates in the dog. Suspensions of macrophages, which had been largely freed

[8] Rich and McKee (114) have recently brought forward striking evidence of the important rôle of the neutrophilic leucocyte in the early localization and destruction of pneumococci. These workers found that avirulent pneumococci injected into rabbits previously made leucopenic with benzol produced in general the same kind of infection as did virulent pneumococci in normal animals.

[9] C. G. Loosli in a study of the origin of the macrophages occurring in the pneumonic exudate of experimental pneumococcus pneumonia.

from neutrophiles, were employed. Tables 1 and 2 show that the macrophages not only engulf pneumococci more actively than do the

TABLE 1

*Comparison of the in vitro antipneumococcal activities of macrophages and polymorphonuclear leucocytes using a concentrated macrophage suspension*

A. Phagocytosis

Opsonic fluid 0.3 cc. + cell suspension 0.1 cc. + pneumococcus suspension 0.03 cc.

| OPSONIC FLUID | EXAMINATION AT INTERVALS OF | DEGREE OF PHAGOCYTOSIS BY | |
|---|---|---|---|
| | | Macrophages | Polymorphonuclears |
| | *hours* | | |
| Normal dog serum | 1 | +++ | ± |
| | 2 | +++ | ± |
| | 3 | ++ | ± |
| | 5 | ++* | 0† |
| 1:5,000 antibody solution | 1 | +++ | ++ |
| | 2 | +++ | +++ |
| | 3 | ++++ | +++ |
| | 5 | no pn. | +++ |
| 1:1,000 antibody solution | 1 | ++++ | +++ |
| | 2 | ++ | ++++ |
| | 3 | ++++ | +++ |
| | 5 | no pn. | +++ |
| Pleural fluid of immune dog | 1 | +++ | +++ |
| | 2 | +++ | +++ |
| | 3 | +++ | +++ |
| | 5 | no pn. | +++ |

Degrees of phagocytosis: ±, an occasional intracellular pneumococcus; +, appreciable phagocytosis but majority of pn. extracellular; ++, about as many pn. in cells as free; +++, majority of pn. in cells; ++++, all or practically all pn. in cells.

* Pneumococci beginning to grow.

† Pneumococci growing abundantly.

Note: Macrophage suspension = 80 per cent macrophages and 20 per cent polymorphonuclears. Polymorphonuclear suspension = 96 to 98 per cent polymorphonuclear leucocytes.

polymorphonuclears but show a considerably greater ability to digest these microörganisms. Such differences were most pronounced in the presence of opsonic fluid of relatively low concentration.

DEPENDENCE OF MACROPHAGES ON OPSONINS. The question as to whether macrophages in either normal or immune animals are as dependent on the presence of opsonic fluids for their phagocytic activity toward pathogenic microörganisms as are the neutrophilic leucocytes

TABLE 2

*Comparison of the in vitro antipneumococcal activities of macrophages and polymorphonuclear leucocytes using a concentrated macrophage suspension*

B. Intracellular digestion

Opsonic fluid 0.3 cc. + cell suspension 0.1 cc. + pneumococcus suspension 0.03 cc.

| OPSONIC FLUID | EXAMINATION AT INTERVALS OF | DEGREE OF INTRACELLULAR DIGESTION BY | |
|---|---|---|---|
| | | Macrophages | Polymorphonuclears |
| | *hours* | | |
| Normal dog serum | 1 | 0 | 0 |
| | 2 | + | 0 |
| | 3 | ++ | 0 |
| | 5 | +++ | 0 |
| 1:5,000 antibody solution | 1 | + | + |
| | 2 | ++ | ++ |
| | 3 | +++ | ++ |
| | 5 | C | +++ |
| 1:1,000 antibody solution | 1 | ++ | ++ |
| | 2 | ++ | ++ |
| | 3 | ++++ | ++ |
| | 5 | C | +++ |
| Pleural fluid of immune dog | 1 | ++ | + |
| | 2 | ++ | ++ |
| | 3 | +++ | + |
| | 5 | C | +++ |

Degrees of digestion: +, definite beginning; ++, about as many being digested as whole; +++, more partly digested than whole; ++++, only fragments of pneumococci; C, none seen (complete).

is one which has received a good deal of study. Briscoe (58), Bloom (115), Lowenthal and Misch (116), Lucké et al. (117) investigated the phagocytic activity of macrophages for both normal and specifically immunized animals for a number of kinds of bacteria and alien erythrocytes and found that in the absence of opsonins the macrophages ex-

hibited little phagocytic activity. We (80) tested macrophages from highly pneumococcus-immune dogs and found that they had no ability to engulf virulent pneumococci without the presence of opsonic fluids.

On the other hand, certain authors have brought forward evidence to indicate that during the course of infection or as a result of immunization, the macrophages may be so sensitized as to react more energetically toward the specific microörganism than do the cells of the normal animal. Lurie (118) found that bovine tubercle bacilli were phagocyted and destroyed much more quickly by the macrophages in the tissues of rabbits and guinea pigs previously infected with this microörganism than was the case in normal animals. Furthermore, he made comparative studies in vitro of macrophages from normal and tubercle-immune animals and found that the immune-animal macrophages were more actively phagocytic, not only for tubercle bacilli but also for other bacteria and inert particulate matter (119). This effect appeared to be independent of the opsonic properties of the serum. The fact that the immune body macrophages possessed a generally increased phagocytic activity suggests the possibility that they may represent a more mature type of cell than those first mobilized at the site of the primary infection. In an earlier investigation of the same general nature Clawson (120) was unable to find that the macrophages from rabbits infected with or immune to a tubercle bacillus of low virulence (B.C.G.) exhibited any greater phagocytic or digestive activity for this microörganism than did normal rabbit macrophages. He observed furthermore that immune rabbit macrophages were dependent on opsonic action especially for their ability to digest tubercle bacilli. Other workers, Rich and Lewis (121), and Aronson (122), have shown that macrophages from tubercle-immune animals, when freed from their native fluids, were more sensitive to tuberculins than were the cells from normal animals.

It seems quite possible that altered cellular reactivity as a result of exposure to invading microörganisms may depend on the kind of bacteria as well as on the animals species employed. Coggeshall (123) was unable to detect any evidence of increased sensitivity to the pneumococcus or its products in dogs recovered from experimental pneumonia or vaccinated with this microörganism, whereas Bull and McKee (124) found that rabbits could be highly sensitized to the pneumococci.

Reactions to the tubercle bacillus. While the cellular changes described in the inflammatory exudate of pneumococcus pneumonia in the dog seem to be in general the same as those occurring in response to infection with a number of other microörganisms, both in the lungs

and elsewhere, lesions produced by certain bacteria exhibit different and characteristic variations in their evolution. The acid fast bacteria and, in particular, tubercle bacilli, are in this group. The initial response of the lung to infection with this microörganism is a mobilization of neutrophilic leucocytes as described by Vorwald (125), Maximow (126) and others. These cells phagocyte the bacilli but are unable to digest them and either degenerate or are engulfed by mononuclear phagocytes which accumulate at the site of the slowly growing lesion. These cells, monocytes and macrophages, come from the blood and local tissues. Finally lymphocytes appear in increasing numbers around the periphery of the inflammatory process. The tubercle bacilli are engulfed by the large mononuclear phagocytes and undergo digestion within these cells (127) (70). Sabin and Doan (128) observed that while the macrophages destroyed the ingested bacilli the monocytes simply held them. Other workers do not make this distinction. As the lesion evolves the macrophages become the predominant cell and are designated as epithelioid cells by workers in this field who describe them as large mononuclear phagocytes containing segregated fragments of digested tubercle bacilli. The next stage in the lesion is the formation of giant cells presumably by fusion of epithelioid cells (129) (127) but some believe that they are formed by division of the nuclei within single epithelioid cells (130).[10] The functional state of such giant cells as regards phagocytic activity is not clear. In general the formation of the tubercle is analogous to that of other granulomas.

While the macrophage appears to be able to digest a great variety of living agents, there are certain pathogenic microörganisms which it can engulf but is incapable of destroying. Leishman Donovan bodies (131), lepra bacilli (132, 133), Tr. Cruzii (134), are all actively taken up by the macrophages where they remain in a viable state for relatively long periods and in some instances multiply within the cell.

Intravascular phagocytosis in the lung. The rôle played by the lung in the removal of different kinds of intravenously injected particulate material has been studied by a number of investigators. While there appears to be little question that considerable quantities of such varied substances as bacteria (135), manganese dioxide (136) and silica (137) may be held in the lung capillaries, the mechanism of this action is by no means clear. Hopkins and Parker (135) thought that injected hemolytic streptococci were phagocyted by the endothelial

[10] Gardner (88) believes that the giant cell in the silicotic nodule is formed by nuclear division.

cells of the pulmonary capillaries but could not be sure of this. Likewise Drinker and Shaw (136) observed particles of manganese dioxide in the capillary endothelial cells but found that the bulk of this material was held only temporarily in the lung (138). Gardner and Cummings (137) noted that many particles of injected silica were trapped in the lung capillaries and then phagocyted by large mononuclear cells but they saw particles within giant cells in the alveolar walls. Mole (139) also observed marked phagocytosis of stained (marked) red blood cells in rabbits' lungs but could not be certain of the origin of the phagocyting cells. Neither Cappell (61) nor Westhues (140) could detect any phagocytic activity of the endothelial cells of the pulmonary capillaries for India ink, or collargol. The experiments of Wislocki (141) contribute valuable information on this subject. He found that considerable quantities of finely suspended India ink of a particulate size of 1–3$\mu$, injected intravenously were caught in the lung capillaries but not in the systemic capillary system except the liver and spleen, which suggests that retarded blood flow in the lung plays an important rôle in this process. Wislocki believes that phagocytosis plays no rôle in the trapping of carbon in the lung which material he observed to be present in the capillaries in small plugs. After a time, mononuclear phagocytes collected around the carbon and began to engulf it but the endothelial cells took no part in this process. Thus the evidence presented and that of other workers not cited, indicates that the capillary endothelium of the lung exhibits little or no phagocytic activity for circulating foreign particles.

Phagocytosis by the epithelial cells of the bronchial mucosa. Whether the epithelial cells of the bronchial tree possess any ability to engulf foreign material is another subject of controversy. The majority of workers believe that these epithelial cells are non-phagocytic (11, 59, 38, 96, 9). Gardner and Cummings (94) found no asbestos particles in the walls of the trachea and bronchi even after two years of constant experimental exposure to this material.[11] However, certain investigators, on the basis of carefully conducted experiments, have come to the opposite conclusion. Ropes (142) found that the vitally stained cuboidal ciliated cells of the lungs of rabbits exposed to carbon dust contained many carbon particles. The long columnar cells showed little or none of this material and no phagocytosis by any of these cells

[11] Tchistovitch (71) found that neither the living cells of the frogs' lungs nor those of the swim bladder of the fish exhibited phagocytic activity for particulate matter.

was observed if the India ink was injected in fluid suspension. Duthrie (143) considered that he had demonstrated phagocytosis of carbon by the cells of the bronchial walls but his work was not nearly as convincing as that of Ropes. Smith, Willis and Lewis (144) studied tissue cultures of chick embryo seeded with tubercle bacilli and observed phagocytosis of the bacilli by the non-ciliated epithelial cells of the bronchi and the lining cells of the alveoli. Carlton (145) carried out a well controlled study of the phagocytic activity of the columnar epithelial (nonciliated) cells of the uterine horns and vagina in the rabbit. He was able to demonstrate that these cells engulfed graphite, staphylococci and carmin particles. Castaneda (146) has recently shown that after the intratracheal injection of typhus Rickettsiae the bronchial epithelium becomes parasitized with these microörganisms. The foregoing experiments indicate that under special circumstances and in special loci, epithelial cells may exhibit phagocytic activity. However, regardless as to whether or not certain cells of the bronchial epithelium are capable of phagocytosis, it seems evident that they play a very insignificant rôle in the removal of foreign material by this means.

Significance of phagocytosis in the lung. While the process of phagocytosis represents the most effective means the body possesses for disposing of foreign matter which has penetrated into the terminal areas of the lung, it fails in many instances to protect the body against the noxious action of such extraneous material. This is particularly true of those substances which are not susceptible to the digestive enzymes of the phagocyting cells. It is not inconceivable that the transfer by the macrophages of the irritating dusts (silicates) and lipoid material from the alveoli to the parenchymatous tissues may ultimately result in greater injury to the lung than if such substances were allowed to remain in the air sacs where some possibility of their expulsion exists. However, the progressive accumulation of particulate material in the alveoli, even though non-irritating, would undoubtedly lead finally to serious interference with the respiratory function of the lung. The protection afforded by phagocytosis against invading microörganisms is in general much more adequate since the phagocyting cells possess enzymes which are capable of digesting most kinds of bacteria. In some instances, when the virulence of the microörganism is relatively low or the numbers of implanted bacteria even though highly pathogenic, are very small, destruction of the bacteria may be accomplished entirely by the neutrophilic leucocytes which are the first to be mobilized at the site of infection (18). Even when the infection becomes well established

it is the polymorphonuclear leucocyte which constitutes the body's first line of defense against the pathogenic invaders. This is particularly true in the acute pulmonary infections. The neutrophiles act to check the spread of the infecting bacterium until the more slowly accumulating macrophages take over the function of phagocytosis and digestion. Since these cells possess more potent intracellular enzymes than do the neutrophiles, they will, under favorable circumstances, in conjunction with the humoral immune substances, bring about either complete destruction of the invaders, as in pneumonia, or sharp localization of the infectious process characteristic of the favorably progressing tuberculous lesion.

The important rôle played by the macrophage in the process of recovery from infectious disease has been demonstrated by a number of investigators; Gay and co-workers (147, 148, 149) for the hemolytic streptococcus, Cannon et al. (150, 65, 151) for staphylococcus and B. coli, Robertson and associates (67, 68, 69, 152) for the pneumococcus, Maximow (153), Sabin and Doan (128), Lurie (70), and others for the tubercle bacillus, Taliaferro and co-workers (54, 154, 155) for protozoan infections, to mention only the most extensively studied pathogenic microörganisms. The only generally recognized mechanism by which the macrophage causes destruction of these disease producing agents is that of phagocytosis and intracellular digestion. Gay (147) found that extracts of macrophages and supernatant fluids from macrophage exudates would cause lysis of hemolytic streptococci. But this effect has not been shown for other pyogenic cocci. It is not unlikely that the free macrophages are capable of producing other substances which affect bacteria outside the cells but little definite information is available on this subject.

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# THE BLOOD SUGAR: ITS ORIGIN, REGULATION AND UTILIZATION

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*1. Carbohydrate Metabolism as a Dynamic Balance.* As will appear from the evidence to be reviewed, it is well established that sugar is supplied to the blood by the liver when it is not forthcoming from the gastro-intestinal tract or other exogenous sources. The blood sugar is chiefly utilized in the extra-hepatic tissues. It is also known that in every species of animal and under all experimental conditions in which it has been done the removal of the liver leads to a rapid, profound, and fatal hypoglycemia, which can be alleviated by the continuous administration of relatively large amounts of dextrose. It may therefore be stated as axiomatic that the blood sugar level represents a dynamic balance between the rate at which sugar is entering the bloodstream from the liver and from any exogenous source, and the rate at which it is being removed from the blood by the tissues of the body. Thus a rise in the blood sugar level may result either from an increased rate of sugar supply or from a decreased rate of sugar utilization, or from both together. Conversely, a fall in the blood sugar level may be due to decreased supply, increased utilization, or both. Nor is it possible to tell, from the mere change in blood sugar level, which factor is responsible unless one factor is controlled or eliminated while the other is observed. These considerations also apply to the various other criteria of carbohydrate metabolism with which we shall deal.

This simple but fundamental conception dates back to Claude Bernard. Nevertheless it seems necessary to emphasize it here from the outset, for it has all too often been ignored in the development of some of the most widely accepted views of carbohydrate metabolism. While the concept of the dynamic balance may seem sufficiently obvious when applied to the blood sugar level as above, it has often been overlooked in the consideration of such matters as the D:N ratio, the respiratory quotient, ketosis and carbohydrate balance studies. In discussing

these topics we shall, therefore, have frequent occasion to allude to this basic error, and to attempt to avoid it in our own interpretations.

PART I. ORIGIN OF THE BLOOD SUGAR. *2. The Site of Formation of the Blood Sugar.* The secretion of sugar into the blood by the liver of the fasting animal was demonstrated by the brilliant pioneer work of Claude Bernard (10). The inadequate chemical and physiological methods available to him and to his contemporaries initiated an involved controversy which ultimately partly obscured his original conceptions (289). Subsequent observations on the development of hypoglycemia following hepatectomy in birds (139) (190), and after total abdominal evisceration in mammals (172) (207), again directed attention to the liver as the source of the blood sugar. It remained for F. C. Mann (177) to devise the first practical method for complete hepatectomy in dogs. Mann's three-stage operation was simplified to a two-stage procedure by Markowitz and Soskin (183) (240), in which form it has been possible to apply it to dogs, cats, rabbits, guinea pigs and monkeys (181). More recently, Markowitz, Yater and Burrows (184) have described a feasible one-stage method of hepatectomy which, with slight modification, makes total abdominal evisceration of the dog (255) and rabbit (195) a relatively simple matter. It is upon these methods that much of the recent progress in evaluating the hepatic and peripheral aspects of the dynamic carbohydrate balance has depended.

The work of Mann and collaborators finally established the liver as the prime factor for the maintenance of the normal blood sugar level. The presence of appreciable quantities of glycogen in the muscles of their hepatectomized dogs during profound hypoglycemia led them to conclude that muscle glycogen is incapable of sufficiently rapid conversion to glucose to play a significant rôle in maintaining the blood sugar level (19). This conclusion was confirmed and extended by Soskin (237) who demonstrated that the known hyperglycemic agents, epinephrin, ether anesthesia, and asphyxia, have no influence whatever on the falling blood sugar level of liverless dogs. More recently Houssay and his associates have found a similar absence of the hyperglycemic action of extracts of the anterior pituitary gland, in hepatectomized toads (34) (119) and dogs (120). Soskin concluded that muscle glycogen is not an available source of blood sugar in the absence of the liver, and that the liver is the sole source of supply for the glucose in the blood in the fasting organism. He pointed out, however, that muscle glycogen might give rise to blood sugar in the intact animal by indirect means;

that is, when lactic acid is produced in excess by the muscles, reaches the liver by way of the blood stream, and is there converted into glycogen. This so-called lactic acid cycle was later investigated and elaborated by Geiger (84) (85) (86), Himwich (109), Cori (42) and others.

*3. Use of the Diabetic Organism in the Study of Gluconeogenesis.* The chief medium of research as regards the precursors of sugar in the animal organism and especially as regards the amount of sugar which may be derived from them, has been experimental diabetes. More specifically, the extra-excretion of sugar in the urine of the depancreatized or phlorhizinized animal following the administration of a known amount of a foodstuff, has been taken as the measure of the extent to which that substance may give rise to blood sugar. The rationale of this method is based upon *evidence* that the diabetic organism cannot store much carbohydrate, and upon the *assumption* that the newly-formed sugar cannot be utilized but is excreted quantitatively into the urine. The empiric usefulness of the information derived from the method has, in turn, tended to raise the latter assumption concerning the nature of diabetes to the dignity of a proven fact. The evidence to be reviewed later shows that the diabetic organism can and does utilize large amounts of blood sugar. This does not entirely invalidate the data obtained in feeding-excretion experiments. It does necessitate a brief consideration of the nature of the diabetic disturbance in order that such data may be safely interpreted.

From the standpoint of the dynamic balance and avoiding post hoc propter hoc reasoning, the hyperglycemia and glycosuria of diabetes may be due to either or both of two possible disturbances: *1*, a loss of the ability to utilize blood sugar, accompanied by a normal rate of gluconeogenesis (the Non-Utilization Theory); *2*, an increased rate of gluconeogenesis accompanied by a normal rate of utilization (the Overproduction Theory).

When these two possibilities are represented in a simple mechanical analogy, as in figure 1, it is readily seen that the addition of an extra quantity of sugar *in either B or C* will result in an extra overflow through the kidney in addition to the excess which is already being excreted. Thus, the more or less quantitative excretion of administered sugar by the severe diabetic does not constitute proof for or against either theory. Similarly the administration of a given amount of a non-carbohydrate precursor of blood sugar will result in an extra excretion of sugar on the basis of either theory. However, here the similarity ends. For, if the non-utilization theory is correct, the extra excretion of sugar must

be a quantitative measure of the extent to which the precursor is converted into sugar. While, if the overproduction theory is correct, there is the possibility of some coincident variation in the rate of utilization so that the amount of extra sugar which is excreted may represent only a portion of that which is derived from the precursor. It must also be remembered that, on the basis of either theory, the extra excretion of sugar following the administration of a precursor will depend upon the capacity of the liver for gluconeogenesis from that particular precursor over and above that which is already proceeding. This is particularly apt to apply in the case of overproduction, where gluconeogenesis from one or more of the endogenous non-carbohydrate precursors may already be near or at its maximum.

*4. Gluconeogenesis from Protein.* (*a*) *In the depancreatized animal.* The origin of blood sugar from ingested carbohydrates or from liver

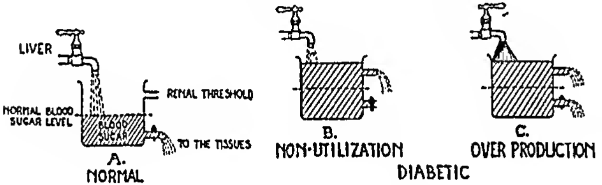


Fig. 1

glycogen resulting from carbohydrate feeding, requires only passing mention. A consideration of the non-carbohydrate precursors of blood sugar in the fed or fasting animal properly begins with the pioneer work of Mering and Minkowski (188) on depancreatized dogs. From the extensive glycosuria exhibited by these animals, and especially from the fact that administered sugar was excreted more or less quantitatively in the urine, Minkowski (191) concluded that depancreatized dogs have lost the power to utilize carbohydrate. He further observed that, for a varying number of days during an intermediate stage of his experiments, the amount of glucose excreted by the animals bore a fairly constant relationship to the amount of nitrogen excreted in the urine. This relationship, which was not affected by varying the amount of protein in the diet, was named the D:N ratio. Minkowski interpreted this ratio to mean that protein was the sole source of the excreted sugar, and that a constant proportion of the protein was being converted

into sugar. It is apparent from our previous discussion of the possible disturbances in the diabetic organism, that Minkowski's conclusions from the glycosuria and the quantitative excretion of administered sugar were not justified. If, however, the D:N ratio were really constant, it would be difficult to conceive of a rate of sugar utilization (except zero utilization) so unvarying in different animals and under different conditions as to make the ratio possible. A critical examination of the constancy of the D:N ratio is therefore in order.

On 31 experimental days, in 9 depancreatized dogs fed on meat, Minkowski obtained D:N ratios which varied from 3.16:1 to 2.62:1, with an average of 2.8:1. The experimental days which he used to establish the average ratio were admittedly selected, since a record of all the experimental days on any single animal would show D:N ratios

TABLE 1

*The days, during the diabetic life of his dogs, which Minkowski (191) used to compute his average D:N ratio*

| DOG | DAYS AFTER PANCREATECTOMY | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | 2 | 3 | 4 | 5 | | | | 9 | 10 | | | | | |
| II | | | | | | | | | | | 12 | 13 | | |
| III | | | | | | | | 9 | 10 | 11 | | 13 | | 15 |
| IV | | | | | | | 8 | 9 | 10 | | | | | |
| V | | 3 | 4 | | | | | | | | | | | |
| VI | | | | | | 7 | | | | | | | | |
| VII | | | | | 6 | | 8 | | | 11 | | | | |
| VIII | | | | 5 | 6 | 7 | 8 | | | | | | | |
| IX | | | | | | | | | | 11 | 12 | 13 | 14 | 15 |

much higher than 2.8:1 to begin with, and ratios which fell progressively below this figure as the exitus of the animal was approached. The high initial D:N ratios were discarded on the basis that they represented the pouring out of preformed glycogen stores. The low D:N ratios towards the end of the experiments were disregarded because of the poor condition of the animals at that time. The reasonableness of these objections to the results of the first and last few days of each experiment cannot be denied. But a closer examination of his data makes it apparent that the experimental days were selected in a much more arbitrary manner than we have been led to believe by those who have trustingly accepted Minkowski's average as a physiological constant.

Thus, the selected data in any given experimental animal began as

early as the second day of diabetes or as late as the twelfth day, and ended as early as the fourth day of diabetes or as late as the fifteenth day. The days reported in some experiments, moreover, are not consecutive, some days being omitted for no stated reason. It must be apparent that any desired average D:N ratio might have been obtained by such arbitrary selection of experimental days, picked from experiments in which the D:N ratios fell progressively from high to low values.

This criticism is supported by other results of Minkowski, reported in the same paper but not included in the figures from which he obtained his average D:N ratio. In comparing the initial D:N ratios obtained from well-nourished and poorly-nourished animals, he recorded ratios of 2.04, 2.43, 1.62 and 2.24 on the third, fourth and fifth days of diabetes in the latter animals. It is difficult to understand why Minkowski did not attempt to correlate these results with the data from which he computed his average ratio. The poor nutrition of these animals might perhaps have accounted for the failure to obtain high initial D:N ratios. But the values uniformly below 2.8:1 obtained on the days in which the approaching demise of the animal was not a factor, and on days which coincided in point of time with some of the experimental days which were used to obtain his average, serve to confirm the arbitrary nature of the average D:N ratio at which he arrived. This indication of the inherent defects in Minkowski's work is not intended to cast aspersions on his integrity as a physiologist. It must be remembered that Minkowski, working before the days of insulin, had to deal with acutely diabetic dogs suffering from the effects of a recent anesthetic and operation.

Pflüger (209), Embden (71) and others subsequently reported that they had failed to obtain fixed D:N ratios at the Minkowski level. Their work was criticized on the assumed grounds of the poor condition of their animals, or of incomplete pancreatectomy. Such criticism, however, cannot be leveled at the work of Macleod and Markowitz (171) who used depancreatized dogs maintained in excellent condition by the use of insulin. After the withdrawal of food and insulin from such animals (which by subsequent post-mortem examination were shown to be completely depancreatized) they obtained D:N ratios far below 2.8:1, after the first few days of the experiment had elapsed. Chaikoff and co-workers (37) reported similar results and found (as noted by Minkowski) that the D:N ratio was generally higher in fat than in lean dogs, and varied decidedly in the same animal, according to its nutritional condition at the time of the experiment.

In 1930 Rapport (217) reviewed the extensive literature on the D:N ratio in addition to the above, and was not able to reconcile the large variations which had been reported. In the same year Soskin (239) published a comprehensive re-investigation of the D:N ratio in depancreatized dogs, using the advanced technique made possible by the discovery of insulin. This work was done on depancreatized dogs, completely recovered from operation by the use of insulin, and presenting well-healed and non-infected wounds. The animals were maintained on a low-calory, protein diet, and the absence of islet tissue was verified by post-mortem examination. In contrast to Minkowski's animals, they survived the withdrawal of insulin for as long as 5 weeks, during which they usually remained bright and active although losing weight. The observations on the D:N ratio comprised 138 unselected days in 10 dogs, in contrast to Minkowski's 31 selected days in 9 dogs. The distribution of the D:N values obtained was as follows:

TABLE 2

*Distribution of D:N ratios during 138 unselected days, in 10 depancreatized dogs (Soskin (239))*

| | RANGE OF THE D:N | | | | | TOTAL DAYS |
|---|---|---|---|---|---|---|
| | Over 3.16 | 3.16-2.62 (Minkowski's range) | 2.61-2.00 | 1.99-1.00 | Less than 1.00 | |
| Number of experimental days on which obtained. | 15 | 33 | 43 | 36 | 11 | 138 |

It may be seen that although some D:N ratios similar to Minkowski's were obtained, there is nothing to indicate that such values have any particular significance. In general the D:N ratio tended to be high at the beginning of each experiment and to show a progressive fall as the animals lost weight and their stores of adipose tissue were depleted. This serves to explain the different D:N ratios reported by previous workers. The fact that some animals maintained D:N values far below 2.0:1 for as long as 18 days, precludes the appellation of "premortal" which some writers have used to avoid consideration of all ratios below the Minkowski level (40).

It is clear that, if Minkowski's interpretation of his ratio is accepted, the progressively lower ratios obtained later in the experiments signify the utilization of increasing amounts of the sugar arising from protein.

If, on the other hand, the low ratios obtained later in the experiments represent the true extent of gluconeogenesis from protein, the higher Minkowski values must mean that sugar is being formed from fatty acid as well as from protein. In either case there remains no basis for concluding that sugar is derived solely from protein, or that none of the sugar so formed is utilized by the diabetic organism. It is permissible to conclude that sugar is derived partly from protein, but it is impossible to say to what extent this occurs.

(b) *In the phlorhizinized animal.* Similar conclusions may be drawn as regards the significance of the D:N ratio of 3.65:1 *formerly obtained* (53) by some investigators in so-called phlorhizin diabetes. There is an added difficulty in interpreting this type of work in that there is no standard for judging the experimental preparation, comparable to histological demonstration of complete pancreatectomy in operated animals. It is obviously fallacious to account for different D:N ratios obtained with phlorhizin in different animals and by different workers (217) by saying that some of the animals were not completely phlorhizinized because they did not yield D:N ratios of 3.65:1. But even if one were to accept the latter figure as correct, one would still have to explain the difference between it and the Minkowski ratio of 2.8:1. There is no factual basis for concluding that phlorhizin alters the biochemical processes in such a manner as to allow a larger proportion of the protein molecule to be converted into sugar. And, if a constant proportion of the protein molecule is convertible, then either the depancreatized animal always utilizes a significant fraction of the sugar derived from protein, or the phlorhizinized animal must be forming sugar from fat as well as from protein. We must add to this the evidence that: (a) Insulin has been obtained from the pancreas of dogs after prolonged and maximal phlorhizin treatment (204). (b) The phlorhizinized dog after nephrectomy is quite normal as regards its blood sugar level, its respiratory quotient, and the rise in the R.Q. following glucose administration (56) (72). (c) The ingestion of sugar by the intact phlorhizinized animal results in the retention of glucose, which has an antiketogenic and protein-sparing action, and causes a rise in the R.Q. comparable to that which occurs in the normal dog (56) (198) (203) (277) (278). (d) Carbohydrate balance experiments on eviscerated phlorhizinized dogs show that the extra-hepatic tissues of these animals utilize sugar at the same rates as do the tissues of normal animals at similar blood sugar levels (251). It is, therefore, quite apparent that the D:N ratio of phlorhizin-treated animals cannot sig-

nify that no sugar is being utilized, or that the sugar which is excreted represents either the partial or total amount which is being formed from protein alone.

*5. Gluconeogenesis from Fat.* We have seen that the D:N ratio does not warrant the conclusion that sugar cannot be utilized by the diabetic organism. It will be shown later that there is ample positive evidence that carbohydrate utilization is not much impaired. The dextrose component of the D:N ratio therefore represents the net remainder, when the extra-hepatic utilization is subtracted from the hepatic blood sugar formation. Once this is granted, it must inevitably be concluded that the breakdown of protein cannot supply sufficient sugar for both excretion and utilization, and that the liver forms sugar from fatty acid as well as from protein. The long controversy on this subject in the past was made possible only by the dearth of positive evidence of carbohydrate utilization, and the fact that the calculations of the "non-oxidation" proponents were always made on the assumption that none of the sugar formed by the liver was utilized in the diabetic organism. The evidence for gluconeogenesis from fat on this basis has been comprehensively reviewed by Geelmuyden (83) and Macleod (170) and will not be discussed here. We will consider the more recent evidence, in which the utilization of sugar is taken into account, or in which it is excluded as a factor by direct observations on the formation of sugar in the isolated liver.

From carbohydrate balance experiments on rats and rabbits, Cori (42) calculated the quantities of blood sugar which the liver would have to secrete in order to account for the epinephrin hyperglycemias which he observed. It was apparent that the liver could not supply the necessary amounts of blood sugar, unless the greater portion of that sugar were derived from fatty acids. Since he considered the latter process unlikely, he marshalled certain evidence from which he concluded that epinephrin hyperglycemia is chiefly due to a decreased utilization of sugar by the muscles. Soskin and co-workers (259) tested Cori's findings and conclusions by using measurements of bloodflow to convert arterio-venous blood sugar differences into amounts of sugar retained by the muscles per unit of time. They could find no indication that epinephrin decreased the utilization of sugar. Similar negative results were obtained when the experiments were repeated with the coöperation of Essex, Herrick and Mann (245). Still more recently, Himsworth and Scott (108) have reported that epinephrin actually increases the utilization of sugar by the peripheral tissues. Although the significance of

the data which they interpreted to mean an increased sugar utilization may be open to question, their results certainly confirm the fact that epinephrin does not cause a decreased utilization of sugar. From these considerations it must be concluded that epinephrin hyperglycemia is a hepatic affair and that, if Cori's above mentioned calculations are correct, they indicate the occurrence of gluconeogenesis from fat in the liver, under the influence of epinephrin.

Recently Young (287) (288), using figures for the sugar utilization of the extra-hepatic tissues of normal and depancreatized animals, which are available from hepatectomy experiments, has calculated that sugar must be formed from fatty acids in the livers of both types of animal.

It is apparent that the indirect demonstration of gluconeogenesis from fat is not difficult, once the masking effect of the simultaneous utilization of the formed carbohydrate is taken into account. Those who have not accepted the evidence that the diabetic organism utilizes carbohydrate have, of necessity, denied the possibility of gluconeogenesis from fatty acid on the basis of indirect evidence, involving *a*, the D:N ratio; *b*, ketosis, and *c*, the respiratory quotient. It is therefore necessary to anticipate the discussion of carbohydrate utilization, with a consideration of the alternative explanations of these metabolic criteria.

*a. The D:N ratio.* The administration of fat to experimentally diabetic animals has usually not resulted in sufficient excretion of extra-sugar to indicate gluconeogenesis from fatty acids, when the calculations were made on the basis of the classical interpretations of the D:N ratio (217). This is not surprising when it is remembered that these interpretations, by their very nature, practically exclude the possibility that such calculations might yield positive results. Even so, extra sugar might still be excreted if the experimental animal could make additional amounts of sugar over and above that which it is already forming from endogenous protein and fat, including the amount which is being utilized during the experiment. But this involves the unwarranted assumption that the capacity of the liver for gluconeogenesis from fat has not been reached before the fat is administered. The fact that this is not the case for protein has no bearing, for it happens that fat is the only stored food substance present in practically unlimited amounts as far as the daily requirement of the body is concerned. It might therefore be expected that it would be used to capacity when the liver is forming sugar at an uncontrolled rate. From the practical

standpoint, the experimental procedure to test the extra sugar excretion involves the administration of fat to the diabetic animal on the fourth or fifth day after pancreatectomy, after the withdrawal of insulin, or after starting phlorhizination. At this time the animal is suffering from acute diabetes with ketosis, and the administered fat makes him even more sick. In certain experiments in which some extra excretion of sugar after fat administration was reported (238) the animals shortly died. In order to obtain positive results by this method it is apparently necessary to exceed physiological limitations, to a degree incompatible with life.

*b. Ketosis.* The ketosis in diabetes and other conditions has been explained in two chief ways. Those who accepted the Minkowski D:N ratio and the orthodox interpretation of the R.Q. have regarded the ketone bodies as abnormal toxic products of incomplete fat oxidation, which accumulate because of the lack of simultaneously oxidizing carbohydrate (111) (225) (232) (284) (285). Others have regarded the ketone bodies as essentially normal intermediate metabolites, appearing during the conversion of fatty acid to sugar, and accumulating in abnormal amounts whenever the rate of gluconeogenesis from fat becomes excessive (83) (170) (206) (239). Parenthetically, it should be mentioned that it is agreed that unimportant amounts of ketone bodies may also arise from certain amino-acids. Beginning with Embden and coworkers (69) many investigators demonstrated that the liver was the chief source of the ketone bodies, and that the fatty liver forms more ketone bodies than one rich in glycogen (151) (216). It was also found that livers from depancreatized or phlorhizinized animals produced more ketone bodies than livers from normal animals (70).

The crucial difference between the two viewpoints outlined above was that the first theory ascribed the ketosis of diabetes to an inability of the extra-hepatic tissues to dispose of the ketone bodies, while the second hypothesis postulated a rate of hepatic formation in excess of the existing normal ability for peripheral disposal. The work of Chaikoff and Soskin (38) strongly supported the latter viewpoint. They showed that the tissues of the eviscerated-depancreatized dog disposed of injected sodium acetoacetate with equal facility as compared to the extra-hepatic tissues of the normal dog, and that the apparent slower rate of disposal after injection into the intact diabetic animal was due to excessive amounts of endogenous ketones with which the tissues of the animal were already dealing. More recently, the same viewpoint has been firmly established by the outstanding work of Mirsky and

collaborators. Using a ketogenic extract of the anterior pituitary gland, they demonstrated that the ketones so formed arise only in the liver (192), that insulin which suppresses this ketosis does so by acting on the liver and not on the extra-hepatic tissues (193), and that the rate of disposal of the administered sodium salt of β-hydroxybutyric acid by the extra-hepatic tissues was independent of the amounts of sugar being utilized by those tissues (196) (197) (273). This and other evidence (62a) make it no longer possible to regard ketosis as indicating a lack of carbohydrate oxidation by the muscles. It is clear that in diabetes the ketone bodies, like the blood sugar, are utilized by the extra-hepatic tissues, but are produced by the liver at rates which exceed the power of the tissues to dispose of them.

*c. The respiratory quotient.* The non-protein R.Q. of about 0.7, which is usually obtained in both the fasting and the diabetic states, has been interpreted to mean that fat alone is being oxidized. Since the conversion of fat to carbohydrate occurring simultaneously with the oxidation of fat would result in lower R.Q. values, it has been concluded that this conversion does not occur. But, it is becoming increasingly more evident that the respiratory quotient of the whole body, like the D:N ratio, cannot be regarded as the index of a single process. The orthodox interpretation of the non-protein R.Q. of about 0.7, involves the tacit assumption that the only vital processes (aside from protein catabolism) which are in progress and which ultimately consume oxygen and give rise to carbon dioxide, are those associated with the oxidation of fat. Yet there is very satisfactory evidence that other processes which require oxygen or yield carbon dioxide are taking place under those conditions. It is generally agreed, for example, that the brain derives its energy solely at the expense of carbohydrate, and yields an R.Q. of about 1.0 at all times (3) (5) (57) (110) (156). This high R.Q. must be balanced by a correspondingly low one, if the composite R.Q. of 0.7 is to mean anything at all. Indeed, this principle of different R.Q.'s forming a composite total R.Q. is generally used to interpret an R.Q. over 1.0, on the basis that the oxidative R.Q. is being elevated by a simultaneous conversion of carbohydrate into fat (220). But there is no evidence that the high R.Q. due to the latter process cannot be one of the components of any R.Q. under 1.0 (185) (217).

Similarly, Cathcart and Markowitz (35) and others have shown that the oral administration of 50 grams of glucose to the fasting human causes a leisurely rise in the R.Q. to values somewhat less than 1.0, while the administration of equivalent quantities of sucrose, galactose,

levulose or dihydroxyacetone causes a prompt rise in the R.Q. to values above unity. Since the rates of absorption of the latter substances from the gastro-intestinal tract cannot account for the difference, and since it is known that they must be converted to dextrose by the liver before they can be utilized by the extra-hepatic tissues, it is clear that even such relatively simple foodstuffs do not yield R.Q.'s which represent only the process of oxidation.

Much has been made of the fact that the R.Q. of the whole mammalian organism has not very often been found to fall below 0.7. Indeed it was formerly customary to ascribe any lower R.Q. to some undetected fault in technic. More recently, however, members of the Lusk school have themselves obtained such low R.Q.'s (63) (100) and have reviewed other instances in the literature which are similarly free from technical criticism (100). Some of these low values were obtained in normal human subjects under special conditions of feeding, for example, on high fat intakes before the subjects became acclimatized to the abnormal diet. This is significant because the customary feeding habits of man and of animals have resulted in rather arbitrary conventions as to the number, composition and size of meals, and as to the periods during which R.Q. measurements of the absorptive and post-absorptive states are made. The intake of food is ordinarily spread over a considerable proportion of the 24 hours. This means that all the various oxidations, conversions, etc., which yield the highest and lowest components of the composite R.Q., are usually proceeding simultaneously. Under these circumstances one could hardly expect to obtain anything more than an intermediate range of values for the R.Q. of the whole body.

To succeed in demonstrating a truer range for the component R.Q.'s of the body, on a normal diet, it would be necessary to set the experimental conditions so as to allow the processes responsible for either the lowest or highest component R.Q.'s to predominate temporarily. In other words, it would be necessary "to catch the metabolic processes off balance." This has been done by Werthessen (276) who trained rats to eat their entire 24-hour food requirement within a period of 1 to 5 hours. He found that in the same animal, after such a meal, the R.Q. determined at intervals varied from extremely low to extremely high values. The range of these variations in all his animals was from 0.27 to 1.70! Werthessen concluded that the mechanism of oxidation consists of a series of reactions and that the steps include the conversion

of fat into carbohydrate. Markowitz (personal communication) working with Cathcart, performed this experiment upon himself, and obtained similar results to those reported by Werthessen.

The theoretical R.Q. for the conversion of protein to carbohydrate has been variously calculated as 0.613 (Magnus-Levy—173), 0.632 (Lusk—168) and 0.706 (Geelmuyden—83). The R.Q. for gluconeogenesis from fat has been calculated to be about 0.28 by Pembrey (208) and by Macleod (170). The theoretical R.Q. for ketogenesis from fat may be calculated to range from 0.65 to 0.00, depending upon the number of molecules of $\beta$-hydroxybutyric acid which are supposed to arise from one molecule of fatty acid. The work of Blixenkrone-Møller (14) strongly indicates that the value lies closer to zero than to the higher figure. In view of the composite nature of the R.Q. of the whole body and the evidence that carbohydrate is utilized, the diabetic R.Q. of 0.7 can best be interpreted as the resultant of at least two factors, namely, a low component due to gluconeogenesis (especially from fat) and ketogenesis in the liver, and a high component due to the simultaneous oxidation of a portion of the resulting carbohydrate and ketone bodies by the muscles. There are probably many other less influential R.Q.'s, arising from different organs and tissues, which also enter into the balance. But the predominant influence of gluconeogenesis and ketogenesis from fat in lowering the R.Q. is strongly supported by repeated demonstrations that diabetic animals and humans deprived of their fat stores by prolonged under-nutrition yield R.Q. values far above the "diabetic" level (101) (133) (221) (239).

Since gluconeogenesis and ketogenesis occur in the liver, it would be expected that R.Q. determinations performed on the isolated liver under the appropriate physiological conditions should yield very low values. This is the case. Gemmill and Holmes (87) found that the R.Q. of liver slices from a rat fed on a normal diet averaged 0.79, while that from a rat fed butter averaged 0.58. Stadie and co-workers (259b) observed R.Q.s of about 0.32 in liver slices from the depancreatized cat. Similarly in the perfused livers of normal and depancreatized cats, Blixenkrone-Møller (14) obtained R.Q. values which averaged 0.57 for the normal and 0.37 for the diabetic animals. From other data, including the high D:N ratios which he observed, he concluded that the low R.Q. of the diabetic liver resulted from: *a*, the desaturation of fatty acids; *b*, gluconeogenesis from protein and fatty acids; *c*, the formation of ketone bodies. We may summarize by saying that the above con-

siderations of the respiratory quotient show that it in no way contraindicates carbohydrate utilization in the diabetic organism, or hepatic gluconeogenesis from fatty acids.

*d. From the biochemical viewpoint.* We have seen that, when the utilization of carbohydrate by the normal and the diabetic organism is not ignored, the conversion of fat to carbohydrate can be indirectly demonstrated. The objections to this conclusion, which are also based on indirect evidence (D:N, ketosis, and R.Q.), have been overcome. There are also a number of experiments which favor gluconeogenesis from fat even though the investigators did not take into account the factor of utilization. In these experiments neutral fat or fatty acids were administered to *intact* normal or diabetic animals; or certain hormones or drugs were given to such animals, in an attempt to force excessive gluconeogenesis from endogenous fat stores. The results of these experiments were judged by the increases in carbohydrate content of the liver and muscles of the normal animals, and by the increased sugar excretion of the diabetic animals. As might be predicted from our previous discussions of the dynamic balance and the D:N ratio, these experiments have yielded both positive (2) (36) (39) (112) (170) (200) (238) (264) and negative (20) (137) (217) results. Under the circumstances it is justifiable to place greater weight on the positive than on the negative findings. Moreover, some of the negative results must be qualified in that sugar was found to be derived from lower fatty acids with an odd number of carbon atoms, but not from lower fatty acids with an even number of carbon atoms (54) (64). It is implied that the naturally occurring even-numbered higher fatty acids may be degraded only by $\beta$-oxidation, that only even-numbered lower fatty acids will result, and hence that no sugar can be derived from the naturally occurring fats. As opposed to this it may be pointed out that Jowett and Quastel (136) and Leloir and Muñoz (153), who tested the oxidation products of various fatty acids incubated with isolated tissue slices, concluded that $\beta$-oxidation was by no means the only pathway for fatty acid breakdown. In fact, many possible pathways have been demonstrated including $\alpha$, $\beta$, $\gamma$, $\omega$, and multiple alternate oxidations (32) (236) (282). Furthermore, whatever the chemical steps by which it proceeds, the conversion of the naturally occurring fats into carbohydrate has been conclusively proven in the germinating seedlings of plants (89) (128) (149) (179) (189) (202) (219) (231). The point at issue, therefore, is not whether the process can occur, but whether it does occur in the mammalian organism.

*e. Direct evidence for gluconeogenesis from fat.* It remains to consider the more direct evidence concerning gluconeogenesis from fat, when the utilization of carbohydrate by the extra-hepatic tissues is neither affirmed nor denied, but is excluded from the calculation by the experimental method. We have already mentioned the work of Gemmill and Holmes (25) in which they found very low R.Q. values in the isolated liver slices of butter-fed rats. They also observed a coincident increase in the carbohydrate content of these slices, which was greater than the increase observed in liver slices taken from rats on a normal diet. Haarmann and Schroeder (94) (95) added the sodium salts of butyric acid, β-hydroxybutyric acid, and αβ-dihydroxybutyric acid respectively, to surviving tissues (muscle, kidney, spleen, brain and liver) of cats and dogs. With each substance and in practically all tissues they observed a large production of lactic acid. The simultaneous decrease in the carbohydrate content of the tissue, when it occurred, was significantly less than the increase in lactic acid. In the case of the liver, when oxygen was present there was an increase in the carbohydrate content as well as of lactic acid. It was obvious that the lactic acid could not be accounted for as arising from carbohydrate. The authors considered the possibility that the added fatty acids might have stimulated the production of lactic acid from some other substance, but concluded that this supposition could not be justified. They pointed out that in the brain and liver, for example, they were dealing with tissues which ordinarily produce little or no lactic acid, and which contain no other known precursor of lactic acid. Their work, therefore, yields convincing evidence for the formation of carbohydrate from fat through a lactic acid stage. Very recently, gluconeogenesis from fat in isolated mammalian tissue has again been confirmed by Weil-Malherbe (275), who demonstrated the in vitro formation of sugar from added acetoacetic acid by kidney slices.

Another method by which extra-hepatic utilization of sugar has been excluded, and one which is a step nearer the intact organism, is the perfusion of the isolated whole liver. This method is not easy and it is sometimes difficult to obtain satisfactory preparations (91). Nevertheless a number of competent investigators have carried out reasonably successful liver perfusions as judged by a maintained rate of flow of perfusate through the liver with little or no edema, the continued excretion of bile, and the storage of glycogen. Burn and Marks (31) perfused the glycogen-poor livers of fat-fed dogs and of a depancreatized cat. A large production of acetone bodies and of sugar was observed.

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The pre-existing carbohydrate content of the livers accounted for but a small fraction of the sugar which appeared. The disappearance of lactic acid was ruled out as a factor. As regards gluconeogenesis from protein, Burn and Marks rightly (in view of our previous discussion of the D:N) rejected the use of any of the orthodox values for the D:N ratio. Instead, they calculated that if all the carbon in the protein molecule were recombined so as to form dextrose, the ratio of dextrose produced to nitrogen set free in the form of urea and ammonia cannot be greater than 8.3:1. Values for the D:N ratio above this figure would therefore demonstrate gluconeogenesis from fatty acid. Out of a total of 47 determinations of the D:N ratio, 32 exceeded the value of 8.3, and in 7 cases the ratio rose above 17.0.

Blixenkrone-Møller (15) perfused the livers of normal and phlorhizinized cats with sodium butyrate. After accounting for other possible sources of carbohydrate, he obtained D:N ratios ranging from 10.0 to 20.0 or over. Perfusion with sodium succinate yielded D:N ratios as high as 42.0. He concluded that 20 per cent of the added butyric acid was converted into ketone bodies and the remainder went to sugar via succinic acid. Finally, Heller devised ingenious methods (102) to observe the sugar output of the liver in situ, in normal and phlorhizinized cats anesthetized with "Pernocton." After deducting the amounts of carbohydrate which might have come from glycogen, lactic acid and glycerol, he calculated D:N ratios ranging from 5.0 to 18.0 (103).

Figure 2 graphically summarizes the more direct evidence for gluconeogenesis from fat, and indicates the intermediate chemical steps by which it may occur. It is concluded that this process can and does play an important rôle in both the normal and diabetic mammalian organism.

Part II. Regulation of the blood sugar. *6. The Homeostatic Mechanism in the Liver.* The relative constancy of the blood sugar level in the normal animal, under varied conditions of feeding and fasting, indicates the existence of adequate regulating mechanisms. On the other hand, the hyperglycemia and the great dependence of the blood sugar level of the diabetic organism on the kind and amount of ingested food, indicates a profound disturbance in the regulating mechanisms. Claude Bernard was keenly aware of the dynamic balance involved in carbohydrate metabolism; the balance upon which any proper conception of regulation must be based. He clearly stated that the normal blood sugar level represented a precise equilibrium between the rates

that they could not mobilize an additional supply of insulin when sugar was administered. Nevertheless, they yielded normal dextrose tolerance tests, including the Staub-Traugott phenomenon and the hypoglycemic phase (242). But, when hepatectomized dogs, with pancreas intact, were maintained by a constant injection of dextrose just sufficient to keep them at a normal blood sugar level, they invariably yielded markedly diabetic dextrose tolerance curves. It was apparent that the pancreas was not essential to the regulating mechanisms responsible for the normal dextrose tolerance curve, while the presence of the normal liver was essential. This led to observations on the simultaneous blood sugar values of the blood flowing into and out of the liver, in normal and depancreatized dogs, during the course of dextrose tolerance tests. From these and the previous results, it was postulated that, in the presence of a sufficiency of insulin, but not necessarily an extra secretion from the pancreas, the normal liver, as one of its responses to administered dextrose, decreases the output of blood sugar which it has been previously supplying from its own resources. This homeostatic mechanism was later directly proven by Soskin, Essex, Herrick and Mann (246), using blood sugar values and thermostromuhr measurements of bloodflow to calculate the quantitative output and intake of sugar by the intact liver in situ.

The former conception of dextrose tolerance, as compared to the hepatic mechanism which has been demonstrated, is analogous to the difference between an old-fashioned stove and a thermostatically controlled furnace. In the former any excess over the heat requirements is wasted at the expense of extra fuel consumption. In the latter, the rise in temperature above the threshold of the thermostat shuts off the furnace and conserves the fuel until the temperature falls to a level somewhat below the threshold, whereupon the furnace again becomes active. In this analogy, the temperature is equivalent to the blood sugar level and the thermostat-furnace arrangement is the liver. Accordingly, the dextrose tolerance curve and the hypoglycemic phase which often follows it, resemble the fluctuations in temperature above and below the threshold of regulation when an extra quantity of heat is introduced into the system. The characteristics of tho curve depend upon the magnitude of the disturbing factor (the amount of sugar administered), the setting and sensitivity of the thermostat (the endocrine balance), and the capacity of the furnace (the ability of the liver to produce sugar).

From this point of view insulin is merely one of the factors which

determine the threshold of the homeostatic mechanism, and the amount of insulin need not vary to insure adequate regulation. In the normal animal, a rise in the blood sugar level causes the liver to respond by diminishing its output of sugar to the blood. A coincident decrease in gluconeogenesis, by removing a low component of the composite R.Q., can explain part of the rise in the R.Q. which follows sugar administration. The stimulus which elicits the hepatic inhibitory response is the blood sugar itself, and the threshold of stimulation of the hepatic mechanism in a particular animal depends largely upon the endocrine balance, and coincides with the level of blood sugar which that animal habitually maintains.

Although other glands are undoubtedly involved, it may be supposed for the sake of simplicity that the endocrine balance consists of the opposing influences of the hormones of the pancreas and of the anterior hypophysis. When insulin is lacking or when anterior pituitary hormone is present in excess, the liver threshold rises and hyperglycemia results. An excess of insulin or a deficiency of the anterior pituitary hormone lowers the liver threshold so that the pre-existing blood sugar level becomes an adequate stimulus to inhibit the liver, and hypoglycemia occurs. When both insulin and the anterior hypophysis are lacking, as in the "Houssay" animal, the diabetes is ameliorated, and it is possible to obtain most of the criteria of normal carbohydrate metabolism (116) (256), *including normal dextrose tolerance curves* (257), at a glycemic level in between the usual blood sugar levels of hypophysectomized and depancreatized animals. In the depancreatized animal with hypophysis intact, the hyperglycemias caused by sugar administration do not reach the infinitely high threshold of the diabetic liver, which therefore is not inhibited and continues to form and pour sugar into the blood. This lack of hepatic inhibitory effect accounts for the difficulty in influencing the diabetic R.Q. and for the so-called quantitative return of the administered sugar. Thus the metabolic disturbance in diabetes regarded from the standpoint of blood sugar regulation, coincides with the "Overproduction Theory" which we have already been forced to adopt on the basis of the other evidence which we have evaluated. From the same standpoint, other disturbances of carbohydrate metabolism such as those occurring in toxemia (48) (244) (254) are also more readily understood.

The fact that the hepatectomized animal with an artificially maintained normal constant blood sugar level (and with the pancreas and extra-hepatic tissues free to exert whatever regulating powers they

possess) yields "diabetic" dextrose tolerance curves (243),[1] indicates the essential rôle of the liver in this regard. It is not to be supposed, however, that the hepatic mechanism is the only one involved. Glycogen deposition in both the liver and muscle, and an increased utilization of sugar by the extra-hepatic tissues, undoubtedly play their parts. These processes, like hepatic homeostasis, are under the influence of the blood sugar level. Cori and Cori (44) have pointed out that the rate of glycogen deposition depends upon the concentration of sugar in the blood. Soskin and Levine (247) have shown that the rate of sugar

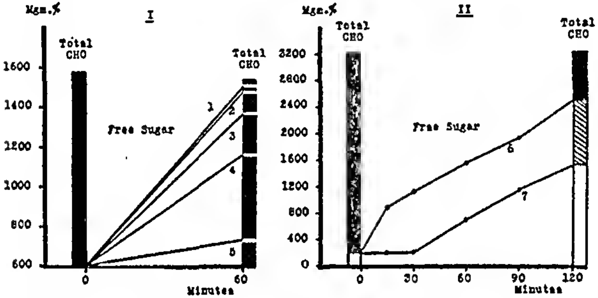


Fig. 3. I. The influence of different amounts of dextrose added to each vessel, upon the appearance of free sugar in liver brei in 1 hour. *1*, no addition; *2*, 5 mgm. dextrose added; *3*, 10 mgm. added; *4*, 20 mgm. added; *5*, 40 mgm. added.

II. A comparison of the rates of appearance of free sugar at different time intervals, with and without the addition of dextrose to liver brei. *6*, no addition; *7*, 20 mgm. dextrose added. The blocks representing total carbohydrate determinations at the beginning and end of each experiment indicate that there was no significant loss of carbohydrate from the system.

utilization by the extra-hepatic tissues varies directly with the height of the blood sugar level. It seems logical to assume that smaller amounts of sugar, especially if they enter the circulation via the portal vein, may be fully compensated for by hepatic inhibition alone. Larger

[1] The liverless animal in which a constant blood sugar level is not being maintained, will exhibit a curve resembling a normal dextrose tolerance test when given an amount of sugar which its tissues are capable of utilizing within 2 hours, namely, 0.5 gram or less per kilogram of body weight. But larger amounts of sugar yield "diabetic" looking curves, showing that the results obtained have little relation to dextrose tolerance as far as regulating mechanisms are concerned.

amounts of sugar will invoke hepatic storage as well. Still larger amounts, which in spite of the above raise the systemic blood sugar level, will bring into play the additional factors of extra-hepatic storage and increased utilization.

It is clear that the fundamental regulation of the blood sugar is an auto-regulation, in which the prime mover is the blood sugar level itself. This is further supported by the work of Soskin, Levine and Taubenhaus (253) on the rate of appearance of free sugar in glycogenolyzing liver brei, with and without the presence of added dextrose. The results are illustrated by figure 3. It may be seen that the sugar level influences the enzyme system concerned with glycogen$\rightleftarrows$glucose, for the rate of appearance of free sugar in the liver tissue is definitely and quantitatively retarded by the addition of dextrose to the brei.

7. *The Pancreas.* The fundamental or primitive hepatic regulation can operate in the complete absence of insulin, as has been demonstrated in hypophysectomized-depancreatized animals maintained without insulin for many weeks (257).[2] In the intact animal, a certain amount of insulin must be constantly secreted to balance the equal and opposite influence of the anterior hypophysis and other factors, in order that the regulation may occur at physiologically normal blood sugar levels. The secretion of additional insulin, over and above that which is already present in the post-absorptive state, is not essential to the primary hepatic regulation, under the particular experimental conditions in which this has been tested (242) (243). However, this does not contradict the considerable body of evidence which indicates that extra insulin is ordinarily secreted as a result of hyperglycemia (82) (99) (124) (125) (147) (148) (262) (296) (297). But it is not entirely certain what purposes are served by this additional insulin. Ricketts (223) has reported that 5 diabetic humans and 2 completely depancreatized dogs, given a dose of protamine insulin on the preceding evening sufficient to maintain their blood sugar at normal levels throughout the following morning if no food was taken, nevertheless yielded diabetic tolerance curves in response to breakfast. On the other hand, Pollack and Dolger (211) performed glucose tolerance tests on 52 diabetic patients, who had received their last dose of protamine zinc insulin 24 hours previously, and obtained normal tolerance curves in many

[2] This effectively disposes of Himsworth's hypothesis (104) (105) according to which the hyperglycemia which follows dextrose administration causes a liberation of "insulin kinase" from the liver, which in turn activates the previously *inert* insulin already present in the blood stream.

instances. Soskin and Hyman, in as yet unpublished experiments closely patterned after those of Ricketts, obtained results similar to those reported by Pollack and Dolger. It seems fair to conclude that, while the secretion of extra insulin is not essential to the intrinsic hepatic mechanism, it acts under normal physiological conditions as a factor of safety in increasing the efficiency of this type of regulation.

As regards the other effects of the provoked extra supply of insulin, it tends to decrease carbohydrate storage in the liver of the normal animal, rather than to increase it (6) (18) (44) (218). It does not cause any increase in the rate of sugar utilization by the extra-hepatic tissues, over that which results from the hyperglycemia itself (248). One way in which it can aid regulation is by increasing glycogen storage in the muscles, for this is one function which practically all agree is facilitated by the administration of additional insulin to the normal animal (6) (12) (13) (43) (45) (248). Extra insulin probably also serves the secondary purpose of decreasing the rate of gluconeogenesis in the face of an oversupply of sugar. This action is a necessary corollary to our consideration of diabetes as a metabolic disturbance in which there is an overproduction of sugar. It is supported, as far as gluconeogenesis from protein is concerned, by the work of several investigators. Janney and Shapiro (129) found that the nitrogen sparing action of glucose in normal animals is increased by the simultaneous administration of insulin. Similarly, MacKay et al. (168a) have reported that, in rats maintained on a diet containing 20 per cent casein and 41 per cent starch, the administration of insulin caused the nitrogen balance to become more positive, whether the food intake was fixed in amount or was given ad lib. Bach and Holmes (4) showed that insulin decreased the gluconeogenesis observed in excised liver slices in vitro, and that this diminution was accompanied by a depression of urea formation. Stadie et al. (259a) have confirmed the fact that insulin inhibits the in vitro oxidative deamination of amino-acids, although they found that the inhibition was not exerted on the l-amino acids, and occurred consistently with the d-amino acids. Mirsky and associates (194) (201) demonstrated that insulin caused a definite decrease in the mobilization of amino-acids from the muscles, probably by stimulating the synthesis of muscle protein. The lowering of the blood amino-acids by insulin has also been confirmed by others (47a) (75a).

*8. The Anterior Hypophysis.* The secretion of the anterior lobe of the pituitary gland opposes the action of insulin in the regulation of the blood sugar. Its opposition, as far as the liver is concerned, is mainly

focussed where insulin exerts its chief effects, namely, on the threshold of regulation of the homeostatic mechanism, and on the rate of gluconeogenesis. After removal of the hypophysis from normal or depancreatized dogs, *well-fed* animals maintain significantly lower blood sugar levels than before hypophysectomy. This is due to the influence of the hypophysis on the threshold of the homeostatic mechanism, which has already been discussed (257). The *fasting* hypophysectomized animal, even when depancreatized, shows a progressive drop in the blood sugar level, and may die in hypoglycemic convulsions (115) (116) (166) (174) (256). There is good evidence that the hypoglycemia of fasting after hypophysectomy is due to a decreased rate of sugar formation by the liver.

The decreased nitrogen excretion of hypophysectomized dogs, and the low D:N ratios exhibited by hypophysectomized-depancreatized animals led Houssay (114) (116), Long and others (160) (165) to conclude that hypophysectomy decreases gluconeogenesis from protein. On the other hand, Soskin et al. (256) showed that meat-fed hypophysectomized dogs had no difficulty in sustaining their blood sugar levels at the expense of the amino-acids derived from the gastro-intestinal tract. But when such animals were fasted, they were unable to catabolize their endogenous body proteins to amino-acids rapidly enough to support a gluconeogenesis sufficient to maintain their blood sugar levels (250). Since orally administered fat did not maintain the blood sugar level of fasting hypophysectomized dogs (256), it was concluded that such animals suffered from a disability to derive an adequate blood sugar supply from both protein and fatty acid, while fed hypophysectomized animals were limited to ingested protein as their chief non-carbohydrate source of blood sugar. The relation of the anterior pituitary to gluconeogenesis from fatty acid is also suggested by the recent work of Neufeld, Scoggan and Stewart (204a). They injected various anterior pituitary extracts as prepared in Collip's laboratory into female mice, and made chemical determinations of the entire carcass of their animals. They found an increase in the total glycogen content, a decrease in the amount of fatty acids present and no change in the nitrogen.

On the other hand, Fisher, Russel and Cori and others concluded that the effects of hypophysectomy resulted from a relatively greater oxidation of carbohydrate (76) (77) (227) and that the administration of pituitary extracts inhibits the utilization of carbohydrate by fixing the body glycogen (the so-called "glycostatic effect") (227) (228) (229). Their conclusions were based upon the calculation of the amounts of

carbohydrate oxidized from R.Q. estimations, and upon carbohydrate balance experiments performed on *intact* fasting animals. We have already discussed the difficulty of accepting quantitative deductions concerning the oxidation of foodstuffs based on R.Q. determinations in the whole animal. Similarly, carbohydrate balance studies done on the intact animal ignore the dynamic balance to which we have referred, for they do not take into account the unknown amounts of sugar formed by the liver during the experimental period.

Accordingly, Soskin and co-workers (249) (252) obtained direct data on the rate of sugar consumption of the extra-hepatic tissues in hypophysectomized dogs, and in normal dogs treated with an active anterior pituitary extract. The variable factor of hepatic gluconeogenesis was eliminated by substituting for it a constant injection of known amounts of dextrose, in abdominally eviscerated dogs. It was found that the extra-hepatic tissues of hypophysectomized dogs utilized sugar at subnormal rates, and that the pituitary extract (which was shown to be capable of maintaining the carbohydrate levels of fasting, hypophysectomized animals) did not influence the rate of sugar utilization by the extra-hepatic tissues of normal dogs. It is therefore, evident that the decrease in carbohydrate levels exhibited by fasting hypophysectomized animals is due to a decreased rate of hepatic gluconeogenesis, insufficient to meet even the reduced sugar utilization of the extra-hepatic tissues. Conversely, the increased carbohydrate levels resulting from the administration of anterior pituitary extracts ("glycostatic effect") is not due to a greater stability of the tissue glycogen, but results from the stimulation of hepatic gluconeogenesis to a rate in excess of the sugar utilization of the extra-hepatic tissues. Crandall and Cherry (46) have confirmed the influence of the hypophysis on hepatic gluconeogenesis in intact non-anesthetized normal and hypophysectomized animals, by means of the London cannula technique. From the blood sugar contents of the inflowing and outflowing hepatic blood, they estimated that the rate of sugar output from the livers of their fasting hypophysectomized dogs was only about 50 per cent of the output from the livers of fasting normal dogs.

The internal secretions of the pancreas and anterior hypophysis are the major opposing forces in the endocrine balance affecting the regulation of the blood sugar level. The thyroid gland and adrenal cortex may be regarded as subsidiary forces, controlled by the pituitary, and acting in the same direction.

*9. The Thyroid.* Clinicians have long recognized the influence of

hyperthyroid or hypothyroid states on carbohydrate tolerance (131) (187) and on co-existing diabetés mellitus in humans (134) (226). In sheep Bodansky (16) found that thyroidectomy caused a decrease in the blood sugar level, while thyroxin administration raised it in normal as well as in thyroidectomized animals. However, since thyroidectomy of the normal or depancreatized dog and cat apparently had little influence on their carbohydrate tolerance, many writers have been led to minimize the rôle of the thyroid in this regard (160) (283) (294). Recently Dohan and Lukens (58) re-investigated the effect of thyroidectomy upon pancreatic diabetes in the cat. The small (though significant) influence which they observed, as compared to the marked effects of hypophysectomy, led them to conclude that the secondary atrophy of the thyroid gland plays a relatively small part in the profound modification of diabetes which follows removal of the hypophysis from the depancreatized animal. It seems probable, however, that the results obtained after thyroidectomy in the common laboratory animals are invalid because the animals do not become really hypothyroid. Marine (180) has demonstrated aberrant thyroid tissue in over 90 per cent of these animals.

Accordingly, Soskin and co-workers (250) studied the problem by administering thyroxin to hypophysectomized dogs. They found that the thyroid hormone maintained the blood sugar level of fasting hypophysectomized dogs not merely at levels which usually occur in the fed hypophysectomized animal, but at levels which are characteristic of the normal dog. This effect could not be ascribed to a further decrease in the already low rate of utilization of sugar by the extra-hepatic tissues of the hypophysectomized dog, for there is good evidence that the thyroid increases the utilization of sugar by the peripheral tissues (92) (186) (195) (215). Thyroxin also increased the urinary nitrogen excretion of the fasting hypophysectomized dogs to that of fasting normal dogs. It was, therefore, concluded that the secondary atrophy of the thyroid gland is partly responsible for the decreased gluconeogenesis which follows hypophysectomy, probably by decreasing endogenous protein → amino-acid catabolism. The influence of the thyroid on gluconeogenesis from protein has been confirmed more recently by Sternheimer (263a) and by Wells et al. (275a) (275b) (275c). The in vitro observations of Klein (140a) of an increased d-amino acid oxidase activity in the liver slices of thyroid-fed rats, also suggest that the effects of thyroid are not confined to the breakdown of protein above the amino acid stage. Part of the confusion which has arisen from the

minor effects of thyroidectomy of (58) and of thyroid administration to (166a) depancreatized animals, may depend upon the possibility that the presence of the internal secretions of the pancreas is essential for the gluconeogenetic effects of the thyroid hormone (241a).

*10. The Adrenal Cortex.* Whether the adrenal cortex influences carbohydrate metabolism primarily (24) (25) (26) (28) (234) or whether its effects are secondary to its control of electrolyte balance (27) (79) (97) (140) (235), is not pertinent to the present discussion. Suffice it to say that total adrenalectomy causes a lowered blood sugar level and decreases all carbohydrate stores in the body (24) (26) (28) (234). It seems likely that the adrenal cortex affects carbohydrate metabolism both directly and indirectly (241a) for, while glucose absorption (1a) and glycogen deposition (1b) may occur normally in salt-treated adrenalectomized rats, nevertheless when such animals are fasted they suffer a sharp decline in the carbohydrate levels of all tissues despite salt treatment (75b) (162a). The lack of adrenal cortical hormone also results in an amelioration of pancreatic diabetes in dogs (163), cats (98) (160) (163) (164) and toads (117), and of diabetes mellitus in the human (15a). These effects are not due to the absence of the adrenal medulla (29) (122) (224) (268) (295). Long, Fry and Thompson (161) (162a) demonstrated that the glycosuria of partially depancreatized rats, which disappears after adrenalectomy, can be made to reappear by the administration of large amounts of a purified adrenal cortical extract. Similar results have been obtained by others (15a) (126a). But, what is perhaps more significant, this same extract increased the liver glycogen in normal rats (138).

Long and Katzin (162) and Russell and Craig (230) have shown that adequate treatment with adrenal cortical hormone prevents the lowering of carbohydrate levels in fasting hypophysectomized rats, or restores them after they have been decreased. The blood sugar and liver glycogen values of the treated rats may exceed the values found in fasting normal rats. However, the adrenal cortex cannot substitute completely for the anterior hypophysis, nor is it essential to all the activities of the latter. Bennett (8) has found that adrenal cortical hormone plus sodium chloride, while maintaining normal muscle glycogen values in fasting adrenalectomized rats, will not do so in hypophysectomized rats. He also showed that, even after complete adrenalectomy, anterior pituitary extract still maintained the muscle glycogen levels of fasting hypophysectomized rats. That the adrenal cortex is not essential for the action of the anterior hypophysis on the

liver is clear from the work of Houssay and Leloir (121) who were able to maintain the diabetic state by hypophyseal extract administration in adrenalectomized dogs. It thus appears that the adrenal cortex stimulates hepatic gluconeogenesis, but is not a necessary intermediary for the anterior hypophysis in this respect. It is possible that the latter stimulates gluconeogenesis from one precursor either directly or through the thyroid gland, and stimulates sugar formation from another precursor through the adrenal cortex.

From the fact that, in some experiments (73) (74) (160), variations in carbohydrate formation caused by experimental variations in adrenal cortical activity have been found to be accompanied by changes in nitrogen excretion, it has been concluded that the adrenal cortex is concerned with gluconeogenesis from protein. There is other work, however, which indicates that the adrenal cortex may also affect gluconeogenesis from fat. Verzar and co-workers (269) (270) (272) have shown that adrenalectomy interferes with the absorption of fat from the gastro-intestinal tract, because of a disturbance in the phosphorylating mechanisms, and have concluded that the adrenal cortex is primarily concerned with phosphate transfer. Recent attempts to confirm these findings and conclusions have been almost uniformly unsuccessful (29a) (41a) (41b) (206a) (226a). However, it seems certain that the adrenalectomized animal suffers from a disability as regards the mobilization of fat from the depots and its transport to the liver. The phospholipids and fatty acids of the blood are decreased after adrenalectomy (286), and various procedures which ordinarily increase the fat content of the liver no longer do so (6a) (169) (271). In addition, Hochfeld (112) has shown that the livers of fat-fed rats treated with adrenal cortical hormone exhibit very significant increases in glycogen, as compared to the livers of untreated fat-fed rats or those of treated rats on a mixed diet. From the standpoint of the dynamic balance, the evaluation of the above evidence as regards the influence of the adrenal cortex on hepatic gluconeogenesis, whether from protein or fatty acids, must await knowledge as to the possible effect of this gland on sugar utilization by the extra-hepatic tissues. It seems likely that peripheral utilization is not an important factor, for Selye and Dosne (231a) have found that while cortin will inhibit the fall in blood sugar of partially hepatectomized rats, it fails to have any effect in completely liverless animals.

*11. The Unknown Factors in Regulation.* Thus far, our account of the regulation of the blood sugar has dealt chiefly with a biochemical

system in the liver, the equilibrium of which is governed by the opposing forces of the pancreas on the one hand, and of the anterior pituitary, thyroid and adrenal cortex, on the other. It has been pointed out that the endocrine glands also affect the activity of the extra-hepatic tissues, although the results of these peripheral actions are apparently not as important to blood sugar regulation as their actions on the liver. However, to say that we have now explained the regulation of the blood sugar level would be an absurd oversimplification of the problem. It is evident, for instance, that the mechanisms which have been discussed do not explain how the endocrine balance is established at the particular height which results in the normal blood sugar level of a particular animal or species of animal. Soskin and Levine (247) have shown that the hyperglycemia of the depancreatized dog enables its extra-hepatic tissues to utilize as much sugar, as the tissues of the normal animal utilize at the normal blood sugar level. But, unless one adopts the purely teleological reasoning of Himsworth (106), it is insufficient merely to state that the height of the blood sugar level is adjusted according to the circumstantial needs of the tissues which utilize sugar. How are these needs communicated to the regulating system? What governs the plane of activity of each endocrine gland? Only fragmentary answers are available. For example, Gerard and McIntyre (87a) working with the thyroid, and others with other glands, have shown that the level of a given hormone in the blood may have an important regulating activity upon the gland which secretes that hormone. It is also possible that the blood sugar level itself plays as important a part in these connections as it does in the homeostatic mechanism of the liver. Himsworth and Scott (107) have compared the effects of a low carbohydrate diet on sugar tolerance and insulin sensitivity with the results of anterior pituitary extract administration, and have ascribed the former to an increased functional activity of the anterior hypophysis. Soskin et al. (258) have reported that the increasing carbohydrate tolerance exhibited by the normal animal which is receiving repeated administrations of dextrose (Staub-Traugott phenomenon), may be explained by a gradual depression of the functional activity of the anterior hypophysis under the influence of a high carbohydrate environment.

It is also possible that the various endocrine glands exert mutually regulatory effects directly on each other, through their internal secretions. This might explain the increased sensitivity of experimental animals to the administration of hormones or glandular extracts, after

ablation of those glands which exert an opposing influence. Perhaps a more striking example of the influence of one gland on another is contained in the work of Young, who climaxed the previous demonstrations of the diabetogenic effect of pituitary extracts (7) (75) (118) by producing permanent diabetes in dogs by the administration of increasing, massive doses of extract (290) (291) (292) (293). At first glance it appeared that he had produced a permanent diabetic state which differed from pancreatic diabetes. It was soon shown, however, that the permanent diabetes of Young was accompanied by an extreme degeneration of the islets of Langerhans of the pancreas (33) (59) (222). It seems likely that the presence of excessive amounts of anterior pituitary hormones in Young's dogs, evoked a correspondingly excessive secretion of insulin, which eventually resulted in an overwork atrophy of the islet cells (10a) (96a) (221a) (241a).

*12. The Emergency Mechanisms.* Finally, it is necessary to mention the parts played by the central and autonomic nervous systems and by the adrenal medulla, in the control of the blood sugar level. These agencies may be regarded as emergency safeguards against blood sugar variations, particularly hypoglycemia, too rapid or too severe to be adequately handled by the regulating mechanisms proper which have already been discussed. It is beyond the scope of this review to discuss these emergency mechanisms in detail. It may be pointed out, however, that their peculiar status is revealed by the fact that adequate regulation of the blood sugar level (except for an increased sensitivity to insulin) ordinarily persists, even after all possible influence of the emergency mechanisms has been eliminated. This has been shown after denervation of the liver (60), denervation or grafting of the pancreas (1) (80) (81) (123) (124) (125) (126) (157), denervation or destruction of the adrenal medulla (23) (29) (152), bilateral vagotomy (210) (214) and total sympathectomy (29) (62). It may be helpful to think of the relationships between the emergency mechanisms, the endocrine glands, and the intrinsic hepatic homeostasis, from the phylogenetic viewpoint. The fundamental or primitive regulation may be supposed to reside in the biochemical processes of the tissue cells. The endocrine glands may represent a step up the evolutionary scale by providing a more sensitive and finely adjusted regulating mechanism, which renders the more highly developed organism less dependent upon its external environment. The emergency mechanisms may be an additional protection against hypoglycemia, for the highly specialized tissues (e.g., central nervous system) of the most highly developed organisms.

PART III. UTILIZATION OF THE BLOOD SUGAR. *13. Significance of "Utilization" Versus "Oxidation."* The term "utilization" is employed throughout this Review, except in the sections relating to the respiratory quotient. It is used to denote the disappearance of sugar from the blood and extra-hepatic tissues, to produce useful energy and heat. The storage of carbohydrate or its return to the bloodstream in altered form are not included within the meaning of the term. The word "oxidation" has been avoided wherever possible, because it carries with it certain traditional physiologic connotations which are no longer acceptable in the light of present-day biochemistry (266). Chief amongst these is the old conception that, by the simple addition of oxygen to their molecules within the tissues of the effector organs, the original foodstuffs may liberate their energy by complete oxidation to carbon dioxide and water. Since the respiratory quotient purports to be a qualitative and quantitative index of this type of reaction, the term "oxidation" is perforce used in connection with it. But, as will appear from the evidence to be reviewed, the oxidative breakdown of the energy materials in the tissues is actually a far more complicated matter, into which the respiratory quotient can give little insight. For this reason, it is hardly more justifiable to speak of "oxidation" as judged from the R.Q., than it is to use the same term to describe the simple disappearance of a substance as determined by chemical balance studies. It would be more accurate to employ the term "utilization" as we have done, and to reserve the term "oxidation" for those rather exceptional instances where the various intermediary metabolites in the degradation of a foodstuff have been chemically identified as oxidation products.

The previous discussion of the respiratory quotient (in relation to gluconeogenesis from fat) allowed the basic postulates of the R.Q. to pass unchallenged, for the time being. In the customary language associated with the subject, it was shown that the R.Q. of the whole body is a composite of many different R.Q.'s arising in the various organs and tissues of the organism. It was pointed out that these individual R.Q.'s were derived from multiple interconversions as well as from purely catabolic oxidations, and it was concluded that the total or average R.Q. could not represent only the kind and amount of foodstuff being oxidized. This suggests that, although the classical physiological interpretation of the R.Q. of the whole animal cannot be upheld, it might be valid if applied to individual organs or tissues. The following discussion of the possible significance of the respiratory quotient, is therefore based upon the evidence obtained under the simplified con-

ditions made possible by the Warburg technique for the study of the respiration of isolated tissues.

*14. Significance of the R.Q. as Judged from In Vitro Determinations on Isolated Tissues.* Under the simplest conditions, a single known substrate can be exposed to a single isolated enzyme system. If the reaction which follows is known to proceed to carbon dioxide and water, without the formation of stable intermediate substances, the amount of substrate which has been oxidized can readily be computed from the oxygen consumed or from the carbon dioxide produced. If a stable intermediate substance of known chemical composition is formed, the R.Q. may be used to calculate the course of the reaction (159). However, it is usually also necessary to determine the amount of original substrate which has disappeared, or the amount of intermediate substance which has appeared, by chemical analysis. When a single substrate is acted upon by an enzyme system and an unknown stable intermediate substance is formed, the difference between the theoretical R.Q. for the complete oxidation of the substrate and the actual R.Q. obtained, may suggest the probable identity of the unknown intermediate (159).

There is no tissue which does not contain a number of substrates and more than one enzyme system. In working with a tissue it is therefore desirable to allow it to approach the zero level of auto-respiration (i.e., to exhaust its own substrates) before the substrate under investigation is added. If the R.Q. of the subsequent reaction agrees with the chemical determination of the disappearance of the added substrate and the appearance of end products, it may then be concluded that the particular enzyme system which it was hoped to engage has operated, and that the supposed course of the oxidative process has been confirmed. It is thus apparent that, even when one can control the other activities of an isolated tissue and is dealing with a single substrate, the R.Q. is merely confirmatory to the information obtained by chemical analysis. When used alone the R.Q. can, at most, merely suggest the probable pathway of a reaction, which must then be demonstrated by chemical means. To illustrate the lack of preciseness of the indications derived from the R.Q., let us suppose that the substrate is hexose, and that no other foodstuff is involved. Let us simplify matters further by considering the possible pathways open to just one of its important intermediary metabolites, namely, pyruvic acid.

Table 3 summarizes the rather formidable list of possibilities, with the experimental or theoretical R.Q. of each. The various observed

total R.Q.'s for pyruvic acid which are cited, have been obtained in different tissues and circumstances, and depend upon the particular combination of the individual reactions favored by the experimental conditions. It is obvious that the total R.Q. of a single tissue, like that of the whole body, is a composite of many possible R.Q.'s. It is also clear that to gain more than the vaguest indication of the fate of the substrate from the R.Q. alone, is a mathematical impossibility. Furthermore, when the chemical determinations have been made, there is little information that the total R.Q. can add, except to act as a check on the possibility that one or more of the end products might have been missed.

TABLE 3

*Possible pathways of pyruvate metabolism*

| REFERENCES | REACTION PRODUCTS | $O_2$ CONSUMED | $CO_2$ PRODUCED | THEORETICAL R. Q. |
|---|---|---|---|---|
| | | *Mm/M. pyruvate* | *Mm/M. pyruvate* | |
| Braunstein and Kritzman (21) | $CH_3CHNH_2 \cdot COOH$ (Alanine) | 0 | 0 | $\frac{0}{0} = 0$ |
| Benoy and Elliott (9) | $C_6H_{12}O_6$ (Hexose) | −0.5 | 0.0 | $\frac{0}{-0.5} = 0$ |
| Long (159) | $CO_2 + H_2O$ | 2.5 | 3.0 | $\frac{3}{2.5} = 1.2$ |
| Elliott and Greig (66). Weil-Malherbe (274). Krebs and Johnson (145) | $COOH \cdot CH_2CH_2 \cdot COOH$ (Succinic A) | 0.75 | 1.0 | $\frac{1.0}{0.75} = 1.33$ |
| Krebs and Johnson (146) (145) | $CH_3COCH_2COOH$ (Acetoacetic A) | 0.5 | 1.0 | $\frac{1}{0.5} = 2$ |
| Long (159) | $CH_3COOH + CO_2$ (Acetic A) | 0.5 | 1.0 | $\frac{1}{0.5} = 2$ |
| Krebs and Johnson (145) | $CH_3COOH + CH_3CHOHCOOH + CO_2$ (Acetic A) (Lactic A) | 0 | 0.5 | $\frac{0.5}{0} = 0$ |

*Observed RQs of pyruvate in various tissues*

| REFERENCES | TISSUE | OBSERVED R. Q. |
|---|---|---|
| Bach and Holmes (4) | Liver | 0.82–1.11 |
| Elliott and Schroeder (68) | Kidney | 1.07–1.24 |
| Elliott, Greig and Benoy (67) | Testis | 1.17–1.41 |
| Elliott, Greig and Benoy (67) | Brain | 1.18–1.23 |
| Long (159) | Brain | 1.28 |
| Elliott, Greig and Benoy (67) | Liver | 1.19–1.76 |

If we now attempt to apply the above to the intepretation of the R.Q. in vivo, there is one further complication which must be mentioned. In the body, the three main foodstuffs or their break-down products

are constantly available, and may be metabolizing simultaneously. It has been shown that amino-acids may yield the same R.Q. of unity as is given by carbohydrate (17). Acetoacetic acid, if completely oxidized, would also yield an R.Q. of 1.0. In view of the limited significance of the R.Q. of a single tissue acting on a single substrate, what possible meaning can be assigned to the composite R.Q. derived from many tissues acting on a variety of substrates? In this predicament the proponents of the R.Q. have sometimes resorted to the argument that, when the R.Q. of the whole body is determined over a sufficiently long period of time, it must represent the resultant of all the R.Q.'s in all the tissues and must therefore ultimately depend upon the chemical composition of the original substrates being oxidized. This ignores:

*a.* The fact that what constitutes a sufficiently long period of time, under various conditions, is difficult to determine; in any case, practical reasons have usually dictated rather short periods of R.Q. measurement in the past.

*b.* The possibility of partial decarboxylation of some of the intermediary metabolites of the original substrate, without further oxidation of the residues, so that the integral of the individual R.Q.'s could never equal the theoretical R.Q. of the original substrate.

*c.* The possibility that some oxygen is used in the formation of storage or excretion products, without the formation of equivalent amounts of carbon dioxide, with the same result as in *b.*

But even if the possible validity of the argument be granted, it is perfectly clear that the composite R.Q. cannot be used to judge the intermediate steps undergone by a substrate, on its way to complete degradation to carbon dioxide and water. In other words, even if we suppose that the R.Q. of 0.7 in diabetes means that the animal is living at the ultimate expense of fat, there is no reason for the further supposition that the fat is being directly and completely oxidized in the extra-hepatic tissues. Thus the R.Q. has no weight against the direct chemical evidence that, in its utilization, fat is converted to hexose and ketones by the liver, and that these intermediates are oxidized by the extra-hepatic tissues. It must be concluded that the determination of the utilization of a foodstuff in the liverless animal by means of chemical balance studies, however incomplete, is a far safer procedure than the attempted calculation of so-called "oxidation" from the respiratory quotient of the intact animal.

*15. Attempts to Determine Carbohydrate Utilization in Intact Animals.* The foregoing conclusion and our previous considerations of the

liver and extra-hepatic tissues as factors in a dynamic balance, are pertinent to the evaluation of certain attempts to determine the utilization of carbohydrate in intact animals. Wierzuchowski (279) used R.Q. measurements to calculate the amounts of sugar oxidized before and after the administration of insulin in 2 normal unanesthetized dogs receiving constant intravenous injections of glucose. According to these calculations, one of the animals oxidized 21.5 per cent of the assimilated sugar before insulin administration, and 27.3 per cent after insulin. But the other animal oxidized 19.1 per cent before insulin, and 19.0 per cent after insulin! The results from the 2 dogs were averaged to arrive at the conclusion that insulin had increased the oxidation of assimilated glucose from 20.3 per cent to 23.2 per cent.

Bissinger and Lesser (13) attempted to compare the disappearance of carbohydrate as determined by chemical analysis with the amounts of carbohydrate oxidized as computed from respiratory data. They used large groups of normal, intact mice, some of which were given intraperitoneal injections of glucose while others received glucose plus insulin. The whole bodies of control, untreated animals, and of the injected animals were minced to determine their initial and final carbohydrate contents, before and after the experimental periods. According to the respiratory data, no glucose had been oxidized up to 30 minutes after glucose alone was administered, while the animals which received glucose plus insulin oxidized 128 mgm. per cent within 30 minutes, and 159 mgm. per cent within 40 minutes. These figures for the insulin-treated animals accounted for 90 to 103 per cent of the carbohydrate which had disappeared by chemical analysis. But, in the animals which received glucose alone, 67 mgm. per cent of carbohydrate (about half the amount which disappeared in the insulin-treated animals) disappeared by chemical analysis during the time that the respiratory calculations indicated that no sugar was oxidized! In accepting these results Cori (42) brushes aside the huge discrepancy with the speculative remark that "It would be of considerable interest to know in what form the sugar is temporarily held in the tissues until it is oxidized to $CO_2$ and water." It might also be asked why a large proportion of the tissue carbohydrate was not also held in the same imaginary form in the mice which had received glucose plus insulin.

The latter author has himself attempted to compare chemical and respiratory computations of carbohydrate utilization in intact rats. The results are summarized in tables on pages 236 and 238 of his Review (42). It is calculated that in normal and adrenalectomized

rats, during 4 hours of glucose absorption from the gastro-intestinal tract, insulin-treated rats oxidized 5 to 12 per cent more of the absorbed glucose than did the untreated animals. This effect of insulin, however, is well within the manifest error of the experiments, for only 85 to 90 per cent of the absorbed glucose could be accounted for by the sum of oxidation, glycogen deposition, and retention in the tissues. In rats given insulin or epinephrin during the post-absorptive state, no true chemical balance was done. Respiratory data were used to calculate the carbohydrate content of the rats at the beginning of the post-absorptive period, while glycogen determinations were performed at the end of the experiment. On this basis it is calculated that insulin doubled the rate of sugar oxidation during the 3-hour post-absorptive period. But it is important to note that, on the same basis, epinephrin also increased carbohydrate oxidation by about 20 per cent. This is significant as to the reliability of the respiratory methods for, in the same Review (p. 188 et seq.), Cori discusses different respiratory work by himself and others which purports to show that epinephrin inhibits the oxidation of carbohydrate! It is apparent that the results obtained in intact animals, using the R.Q. and ignoring the formation of sugar by the liver, merely serve to confirm our previous criticisms of this method of approach.

*16. Utilization of Carbohydrate as Determined by the Disappearance of the Blood Sugar in Liverless Animals.* The rapid disappearance of the blood sugar after removal of the liver from the normal animal has been discussed in the previous section dealing with the site of formation of the blood sugar. The mere withdrawal of sugar from the blood by the extra-hepatic tissues cannot, of course, be regarded as proof of its utilization by those tissues. It is necessary to show that the carbohydrate does not accumulate in the tissues, and that it is not returned to the blood in altered form, *e.g.*, as lactic acid. However, it has been the universal experience that the carbohydrate content of the tissues and the accumulation of lactic acid or any other substance in the blood does not account for the sugar which disappears from the blood of the liverless animal. The rate of disappearance of blood sugar in such animals may therefore be taken as, at least, a rough indication of the utilization of sugar by the extra-hepatic tissues. In view of this, it is significant that the blood sugar disappears after hepatectomy or abdominal evisceration in animals which have been supposed to have ceased utilizing carbohydrate, as judged by the D:N, ketosis and R.Q. exhibited before removal of the liver. Such evidence is available after hepatectomy of depancreatized birds (139), dogs (178) and rabbits (88a) and after

evisceration of phlorhidzinized dogs (61) and of normal dogs fasted to the point of so-called "hunger diabetes" (255). A similar incongruity between the conclusions drawn from the classic metabolic criteria and the disappearance of the blood sugar occurs after hypophysectomy of the depancreatized dog (116) (256) and during prolonged injections of epinephrin in the normal dog (245) (259).

*17. Utilization of Carbohydrate as Determined by Chemical Balance Studies in Liverless Animals.* The groundwork for future chemical balance studies of carbohydrate utilization was laid in the laboratory of H. H. Dale. At that time, practical methods for total abdominal evisceration in the cat were not available. The liver was left in situ with its afferent blood supply tied off. However, the asphyxiated organ with a high free sugar content could still contribute sugar to the blood by seepage into the vena cava. In their later experiments, Dale and co-workers recognized this source of error, and corrected for it by including the changes in sugar content of the liver in their chemical balances. In these experiments, eviscerated spinal cats were given constant intravenous infusions of known amounts of dextrose. The balance was constructed from the amounts of sugar which disappeared from the blood; and from the difference in glycogen and free sugar content between certain muscles removed at the beginning of the experiment, and the corresponding muscles of the opposite leg removed at the end of the experiment. Burn and Dale (30) showed that the increase in oxygen consumption following the administration of insulin to such animals, was usually insufficient to account for more than a small part of the sugar which disappeared from the blood. Best, Hoet and Marks (12) then demonstrated that, when ample sugar was available, a large proportion of the sugar which disappeared from the blood under the influence of insulin was deposited as muscle glycogen.

Finally, Best, Dale, Hoet and Marks (11) measured oxygen consumption and performed the chemical balance on the same animals. They found that, at both high and low blood sugar levels, the sugar which disappeared from the blood during insulin action was equal to the sum of the glycogen deposited in the muscles and the glucose equivalent of the oxygen consumed. These results demonstrated two important facts as regards the validity of chemical balance studies in liverless animals:

1. There is no basis for hypothesizing an unknown intermediate substance retained in the tissues, such as Cori inferred from the work of Bissinger and Lesser.

2. Since the algebraic sum of the carbohydrate which disappears

from the blood and muscles equals the glucose-equivalent of the oxygen consumed, this sum itself can be used as a measure of carbohydrate oxidation.

In accordance with the state of knowledge at that time, Best et al. (11) concluded that the effects of insulin in excess represent an intensification of its physiological effects, including the acceleration of the combustion of carbohydrate. Hence their work has since been quoted as proof that insulin increases the oxidation of carbohydrate. Examination of their original data shows that this is not so. Table 4 summarizes the pertinent figures from the experiments which they themselves selected as being most free from technical criticism. The right hand column is our own recalculation of the amounts of sugar oxidized in milligrams per kilogram per hour, in order to make these values

TABLE 4

*Influence of insulin on glucose oxidation of eviscerated spinal cats (Best, Dale, Hoel and Marks (11))*

| EXPT. NUMBER | ORIGINAL DATA | | | | RECALCULATION |
|---|---|---|---|---|---|
| | Insulin | Weight of cat | Duration of experiment | Glucose oxidized | Glucose oxidized |
| | *units* | *kgm.* | *minutes* | *mgm.* | *mgm./kgm./hr.* |
| 5A | 0 | 3.2 | 50 | 1045 | 392 |
| 5B | 20 | 3.2 | 150 | 2970 | 371 |
| 6 | 30 | 2.6 | 210 | 2595 | 285 |
| 7 | 25 | 2.8 | 250 | 3079 | 264 |

comparable. It may be seen that animal 5 oxidized less sugar after insulin than before. Animals 6 and 7, for which no pre-insulin periods are given, oxidized less sugar after insulin than animal 5 oxidized without insulin.

More recently, Soskin and co-workers have studied the utilization of carbohydrate in totally abdominally eviscerated dogs, by striking a chemical balance from the blood sugar, the blood lactic acid and the muscle glycogen at the beginning of the experiment; the amount of sugar administered in order to maintain the blood sugar at a particular level during the experiment; and the blood sugar, blood lactic acid and muscle glycogen at the end of the experiment.[3] It was found that,

[3] Some of these experiments have since been checked by determining the total carbohydrate content of the muscle instead of muscle glycogen, and lactic acid content of the muscle as well as blood lactic acid. The results were substantially the same by both methods (253a).

between certain limits, the rate of sugar utilization by the peripheral tissues of the normal dog varies directly with the height of the blood

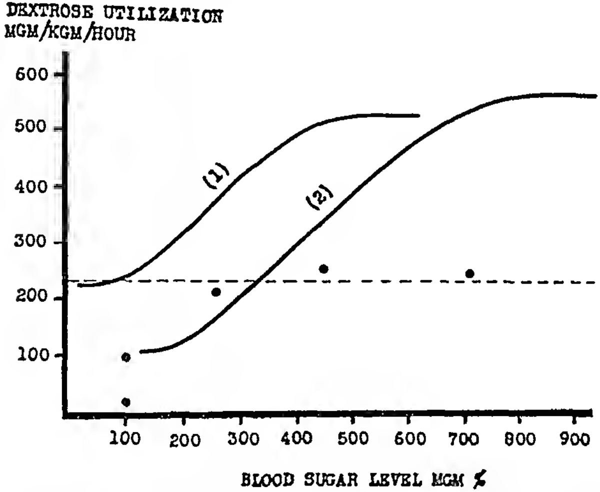


Fig. 4. The relation between the blood sugar level and the rate of sugar utilization, calculated from carbohydrate balance experiments in abdominally eviscerated dogs. The heavy continuous lines are smooth curves drawn through the scatter of individual experimental results for each type of animal. Curve *1* represents the results obtained in the normal dog (247), and in the normal dog treated with insulin (248), anterior pituitary extract (252) or phlorhidzin (251). Curve *2* represents the results obtained in depancreatized dogs (247). The dots represent individual experiments in hypophysectomized dogs (249) (252). The horizontal broken line marks the rate of sugar utilization of normal animals at the normal blood sugar level. This line crosses the diabetic and hypophysectomy curves at a level somewhat over 300 mgm. per cent. Therefore, the uncontrolled diabetic animal, with blood sugar values at or above this figure, utilizes as much sugar as the normal animal at its normal blood sugar level. But the hypophysectomized animal, which ordinarily exists at a subnormal blood sugar level, utilizes much less sugar than the normal animal.

sugar level (247). Within the normal blood sugar range of 60 to 80 mgm. per cent the normal dog utilizes about 240 mgm. per kgm. per hour, which agrees surprisingly well with the fact that Mann (177)

found it necessary to administer about $\frac{1}{4}$ gram of dextrose per kilogram per hour in order to maintain his hepatectomized dogs. Sugar utilization in the completely depancreatized dog also depends upon the height of the blood sugar level (247). The tissues of the depancreatized dog utilize less sugar than those of the normal dog, when both are observed at the same moderate blood sugar levels. Above certain high levels of blood sugar, this difference in rate of utilization disappears. But, most important of all, the diabetic animal at its characteristically hyperglycemic level utilizes as much or more sugar than the normal animal at its normal blood sugar level. This has been independently confirmed by Dye and Chidsey (62a). Thus the disturbance in utilization in diabetes is a relative one, which is compensated for by the hyperglycemia. The administration of insulin, which increases the rate of utilization at any given blood sugar level, also lowers the blood sugar so that no absolute increase in utilization occurs. The various phenomena characteristic of the diabetic syndrome cannot, therefore, be ascribed to a decreased utilization of sugar, and must be due to the overproduction of sugar by the liver as we have previously concluded.

Figure 4 graphically illustrates the utilization of sugar by normal and depancreatized dogs. Observations on the phlorhidzinized (251) and on the hypophysectomized animal (249) (252) are also included. The significance of these latter is discussed in the appropriate sections of this Review, and will not be repeated here. It may be pointed out, however, that the administration of either insulin (248) or of anterior pituitary extract (252) does not affect the rate of sugar utilization by the tissues of the *normal* dog. It may be concluded that the administration of these hormones to normal animals does not change the rate of sugar utilization, because the pancreas and hypophysis are already supplying optimal amounts of their internal secretions.

## SUMMARY

*18. The Mode of Action of Insulin.* By summarizing the various effects of the lack or excess of insulin which have been discussed separately, it is now possible to attempt to picture its influence as a whole. To arrive at a unified conception, it will be necessary to interpolate some unproven assumptions. References are given only for those experimental findings not previously mentioned, but the difference between fact and fancy will be readily distinguishable. It is understood, of course, that one cannot really consider insulin action by itself, for it is but one factor in a balanced endocrine system. Nevertheless, it

is a useful simplification to think of carbohydrate metabolism in terms of insulin, because of the leading rôle which it has played in the historical development of the experimental and clinical aspects of the subject.

An absolute or relative lack of insulin results in a persistent hyperglycemia, with glycosuria. These are the essential and minimal characteristics of the diabetic syndrome. The immediate cause of this syndrome is a failure in the regulation of the blood sugar level. There is no absolute decrease in the rate of sugar utilization by the extrahepatic tissues. But the liver, which normally decreases its supply of sugar to the blood upon the advent of hyperglycemia, no longer responds to this inhibitory stimulus. Instead, it wastes the protein and fat of the body by continuing to form and pour unneeded sugar into the blood. The excess sugar is lost in the urine, carrying with it water and salts, and leading to the secondary symptoms of polyuria, polydipsia, polyphagia, loss of weight, etc. When the rate of hepatic gluconeogenesis from fatty acids becomes sufficiently rapid, the amounts of ketone bodies formed by the liver can no longer be disposed of by the extrahepatic tissues. Ketonemia and ketonuria ensue, the latter involving the loss of fixed base, and leading to acidosis. The latter is probably not responsible for diabetic coma, but signals a metabolic disturbance so severe as to portend coma and death. The ultimate cause of death in diabetic coma is as yet unknown. One may hazard the guess that it is due to the failure of other vital functions of the liver, similar to that which kills the hepatectomized animal when the continued administration of sugar is no longer able to support it.

To change from the negative to the positive definition of its action, insulin is an important factor in the endocrine balance which determines the normal blood sugar level. But it is not essential to the intrinsic hepatic regulating mechanism, for the latter can function to some degree in the complete absence of insulin, providing the opposing endocrine factors are also removed. By the same token, the control of clinical diabetes mellitus by administered insulin does not necessarily mean that this condition is due to a pancreatic deficiency. The introduction of extra insulin into the body can restore the endocrine balance, whether the disturbance be due to a lack of endogenous insulin or to an excess of opposing factors. It is thus possible, in view of the fact that pancreatic pathology is not usually found in diabetes mellitus, that this syndrome may include cases of pancreatic and of pituitary diabetes and, less probably, thyroid and adrenal cortical diabetes. It is even

more likely that certain cases may be termed hepatic diabetes, when the damaged liver fails to respond normally to the presence of a normal amount of insulin.

A consideration of how insulin or the lack of it produces the above effects logically seems to begin with the most firmly established effect of the hormone, namely, the withdrawal of sugar from the blood and its deposition as glycogen in the muscles. This effect can also explain the action of insulin on the liver, for when it is remembered that the liver normally puts out sugar, it will be realized that a decrease in hepatic glycogenolysis is equivalent to an increase in the rate of deposition of muscle glycogen. If it be assumed that the inhibition of glycogenolysis is the primary action of insulin in hepatic regulation, decreased gluconeogenesis from non-carbohydrate precursors may be regarded as a secondary effect. In other words, when glycogenolysis ceases while gluconeogenesis continues, there is a piling up of glycogen in the liver cells to a level which eventually stops further carbohydrate formation. This may occur through the mechanism adopted by Mirsky et al. (199) as the explanation for the antiketogenic effect of liver glycogen, namely, the predominance of the carbohydrate in the substrate competition for available oxygen (65), so that the oxidative breakdown of other foodstuffs is curtailed.

We are now in a position to explain the puzzling difference in hepatic glycogen deposition following insulin administration to the normal and diabetic organisms. When insulin inhibits glycogenolysis in the glycogen-poor diabetic liver, gluconeogenesis continues unchecked until enough glycogen accumulates to stop it. Meanwhile the excess blood sugar has been deposited in the muscles and liver so that (unless too much insulin has been given) the net result is a normal blood sugar level and increased hepatic glycogen. However, any insulin administered to the normal organism is an excess over the optimal amount already present, and causes hypoglycemia. This opposes the direct effects of insulin on the liver by bringing into operation those regulating and emergency mechanisms which result in hepatic glycogenolysis. Hence the usual finding that the glycogen stores of the normal liver are decreased after insulin administration.

It is the hypoglycemia, rather than any unknown stimulating or beneficial effect of excessive insulin on the central nervous system, which also explains the therapeutic results obtained in the insulin treatment of schizophrenia (241). It is generally agreed that nerve tissue has little stored carbohydrate and cannot utilize protein or fat. It must

therefore depend upon the continuous supply of adequate blood sugar to maintain its vital functions. The lower plateau in the S-shaped curve which expresses the relation of the blood sugar level to the rate of sugar utilization, indicates that the latter cannot be depressed below a certain minimal rate by any degree of hypoglycemia. Marked hypoglycemia can therefore drive the supply of sugar from the blood below the amount required for the minimal rate of metabolism compatible with the well-being of the nerve tissues. Thus, insulin "shock" therapy is quite comparable to the other types of shock treatment which have been shown to yield similar therapeutic results.

The fact that the processes which are influenced by insulin have been observed to occur to some extent in the complete absence of the hormone, sheds some light on the nature of its activity. The rate of glycogen deposition depends upon the concentration of the blood sugar (44), but the presence of insulin is not essential (175) (182). Indeed, Dambrosi (49) (50) (51) and Lukens et al. (167) have demonstrated that the restoration of muscle glycogen after depletion by exercise, is just as complete in depancreatized as in normal animals, except that in the former it occurs at a much slower rate. A similar relativity holds for the influence of insulin on the utilization of sugar. In the absence of insulin the depancreatized animal can utilize carbohydrate at any rate of which the normal animal is capable. The difference between the two consists in the fact that the depancreatized animal utilizes less sugar than the normal, when the comparison is made at the same blood sugar level. Above certain high levels of blood sugar, even this difference disappears, and the same rates of utilization prevail whether insulin is present or not. Thus, both as regards glycogen deposition and sugar utilization, insulin modifies the relationship between sugar concentration and the rate of reaction. It accelerates processes which proceed at slower rates in its absence. This activity of insulin resembles that of an activator or an inhibitor of an enzymic process, exerting its influence at some intermediate phase of sugar metabolism, and facilitating the entry of sugar into the tissue cell for either storage or utilization (248).

There have been many unsuccessful attempts to determine the exact point at which insulin acts. Recently, Krebs and Eggleston (144) have concluded that insulin is a limiting factor in the citric acid catalysis of glucose oxidation, in vitro. However, the citric acid cycle has not yet been established as a recognized process in even a single type of tissue (22) (93) (127) (154). Moreover the work was done with the

more likely that certain cases may be termed hepatic diabetes, when the damaged liver fails to respond normally to the presence of a normal amount of insulin.

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# VITAMIN K

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Prior to 1935, several reports mentioning experimentally induced bleeding tendencies in the chicken appeared in the literature (56, 57, 71, 116, 117, 136, 137). While it is now easy to interpret these observations in terms of vitamin K, at the time of their appearance there was a lack of conclusive evidence to distinguish between several possible causes of the bleeding symptoms, such as a deficiency of an unknown dietary factor, a newly recognized effect in the chick of a deficiency of some one of the known dietary factors, or the effect of some toxic or harmful agent. Examples of bleeding tendencies and blood coagulation defects from a wide variety of causes were already known in other species.

In 1935 papers by Dam (58, 59) and by Almquist and Stokstad (24, 25) furnished strong evidence of the existence of a vitamin-like organic factor required by the chick for the maintenance of normal blood clotting power. This factor was named vitamin K by Dam. The early work on vitamin K has been the subject of several reviews (5, 10, 60, 165).

DISTRIBUTION. *Presence in plants.* Green leafy tissue is perhaps the richest natural source of the plant form of the vitamin. As little as one-half per cent of commercial dried alfalfa in the diet of the chick will meet the normal requirements (25). Dried alfalfa has been the initial source used by nearly every laboratory engaged in vitamin K isolation. The vitamin seems to be richest in the photosynthetically active parts of the plant, since the tops of carrots are a good source while the carrot roots contain no detectable quantity (6). Furthermore, the vitamin is more abundant in peas sprouted in the light than in peas sprouted in the dark (66). The inner leaves of the cabbage appear to have only one-fourth the activity of the outer leaves (66).

Among other good sources of the vitamin are spinach, cabbage and kale (66, 72), cauliflower, nettle and chestnut leaves (66). Presence of the vitamin has also been noted in the tomato, hempseed and seaweed (66), and soybean oil (27), as well as in many other minor sources.

*Presence in micro-organisms.* Following early observations on the

development of marked antihemorrhagic activity in foodstuffs during bacterial spoilage (24, 25) and in the droppings of vitamin K-deficient chicks (26), it was found that a number of bacteria, including the common *Bacillus cereus* and *Bacillus subtilis*, synthesize a fat-soluble anti-

TABLE 1

*Antihemorrhage activities of certain micro-organisms**

| PREPARATION | COMPARATIVE POTENCY |
|---|---|
| Alfalfa standard | 1 |
| Bacillus cereus | 23 |
| Bacillus mycoides | 31 |
| Bacillus subtilis | 38 |
| Bacillus vulgatus | + |
| Bacterium aerogenes | 4 |
| Bacterium flexneri | 6 |
| Bacterium friedlanderi | + |
| Bacterium proteus | 15 |
| Bacterium typhosum | 3 |
| Chromobacterium violaceum | nil |
| Clostridium sporogenes | nil |
| Erythrobacillus prodigiosus | 4 |
| Escherichia coli | 3 |
| Myobacterium tuberculosis | 11 |
| Mycobacterium leprae | + |
| Pseudomonas aeruginosa | nil |
| Pseudomonas fluorescens | nil |
| Sarcina lutea | 20 |
| Staphylococcus albus | nil |
| Staphylococcus aureus | 12 |
| Streptococcus lacticus | nil |
| Aspergillus niger | nil |
| Monilia albicans | nil |
| Monilia candida | nil |
| Mucor, Bonar strain | nil |
| Neurospora cytophilia | nil |
| Penicillium, green | nil |

* Adopted from unpublished data (23).

hemorrhagic factor even when cultured on a vitamin-K-free medium (23). Certain dried bacteria were nearly 40 times as potent as a standard dried alfalfa. Other species of bacteria did not possess measurable activity and micro-organisms of the mold, yeast and fungus types were uniformly inactive (23). Some comparative data are given in table 1.

Bacterial action on wet fish meal has been employed in the preparation of vitamin K concentrates (24, 25, 138, 175). Synthesis of the vitamin by bacteria within the intestinal tract of the chick offered an explanation for some cases of spontaneous recovery from, or resistance to, a deficient state (26). The possibility of such synthesis created a necessity for guarding against coprophagy and against bacterial action on the food of the test animals (26). The vitamin has also been found in the feces of vitamin K-deficient, bile-fistula or icteric rats (11).

*Presence in tissues.* In the egg the vitamin appears in the yolk, and the quantity in the newly-hatched chick is influenced by the level in the diet of the parent hen (26). The liver of young chicks (26, 65, 72) and of the rat (110) contains very little vitamin K although that of the dog and hog (72) contains moderate amounts.

The widespread occurrence of the vitamin suggests that its influence on the clotting of blood is only a part of a more general rôle.

DETERMINATION. The biological measurement of vitamin K is based upon the blood clotting power and may be conducted by curative or preventive procedure. Nearly every group of workers has devised a different assay method and has defined a different "unit" of activity, lending confusion to the general comparison of potencies. Many of the "units" had little meaning since they were based on a questionable assumption of standard response by the chick rather than on a reference standard substance. Fortunately, these "units" have been rendered obsolete by rapid progress in the identification of the vitamin and other pure compounds which serve as reproducible standards of activity.

The assay procedure of the Copenhagen group, described by Schönheyder (166) and subsequently improved by Dam and co-workers (67, 70), is so laborious that it has found little use by other workers. The vitamin K-deficient diets used have produced other conditions later traced to additional dietary deficiencies (67, 69).

The method first described by Almquist and Stokstad (27) involved preventive supplementation in the diet and comparison of whole blood clotting times of test chicks with those of positive and negative control groups. The procedure was later improved (16, 22) and based upon the "prothrombin clotting time" method of Quick (149, 152, 159). A linear relation between the reciprocal mean prothrombin clotting time and the logarithm of the vitamin K intake was found useful for the interpolation of assay results (22). More recently (17, 20) assays have been made by oral administration to deficient chicks for four days with determinations of prothrombin time on the fifth.

As compared to simple clotting time measurement, the prothrombin clotting time method was shown to produce less variable data within a test group probably through control of thromboplastin variations (16). The large variability of whole-blood clotting time in the chick is often not closely related to changes in prothrombin level (185). In the case of prolonged whole-blood clotting times there arises also a distinct possibility of deterioration of blood clotting elements during exposure of the blood. The Quick procedure has been further applied and investigated by other workers (1, 112, 140, 146, 175). A 24-hour method based on prothrombin clotting time has also been suggested (155).

The test diet of the California workers (16, 22, 27) has been used with but minor changes by most laboratories (13, 31, 32, 77, 78, 108, 155, 162, 180, 182, 184, 185, 187). This diet leads to normal growth and absence of deficiencies, other than vitamin K deficiency, within the necessary assay period. General severe deficiency occurs after 5 to 7 days when day-old chicks are placed on the diet (27, 149, 185).

A new type of diet, most of which must be heated for 1 week, has been proposed (34); this diet, however, has other deficiencies for chicks so that growth practically ceases. Multiple deficiencies should generally be avoided in biological assays. It is claimed that the diet does not permit bacterial synthesis of vitamin K within the chick or in the droppings.

Other assay methods (32, 78, 182, 187) still retain the more convenient but less accurate whole-blood clotting time. The method of Ansbacher (32) employs depleted chicks with clotting times greater than 90 minutes. Such chicks will often bleed excessively or fatally from the necessary wound, however small, within this period of observation. Severe loss of blood appears to reduce whole blood clotting time independently of vitamin K (53), but has no effect on the prothrombin time (82). The extremely short assay period of 6 hours (32) is sufficient for recovery by the chick to nearly normal blood clotting power and prothrombin level when given a sufficient dose (31, 69, 185). It seems probable, however, that such a short test would be unduly influenced by individual differences in rates of absorption and metabolism of various sources of activity. In fact, the relative activities of certain supplements have been shown to be considerably greater in an 18 hour test than in a 6 hour test (39). On the other hand, those sources of activity which develop a maximum response more rapidly may be more advantageously used in cases of clinical emergency. For a further discussion of assay methods the reader is referred to a review by Dam (61).

Physiology. It was demonstrated by the Copenhagen group (74,

166) that the impaired clotting power of deficient chick blood was due to a reduced level of prothrombin. Later reports (16, 149, 185) have fully supported this explanation. Several other species of birds, namely, the duck, goose, canary and pigeon (73), and turkey (10) are also subject to the same deficiency disease.

No other significant abnormalities have been demonstrated in vitamin K-deficient chick blood when marked hemorrhage is absent (22, 27, 28, 166). Erosions or lesions of the chick gizzard lining were included in the hemorrhagic syndrome in early reports (56, 57, 58, 116) but were later shown to be a result of a different deficiency involving biliary hypofunction (21, 24, 25).

Importance of the antihemorrhagic factor to normal mammals was difficult to demonstrate merely by removing the vitamin from the diet (73). This difficulty may have been caused by failure to deplete reserve stores in the animal, or by bacterial synthesis in the intestinal tract with some absorption (26, 110). Eventually, reports appeared of clearly identified dietary K-avitaminosis in the rat (68, 110), rabbit (63), mouse (143), and even in man (118).

Mammals in certain abnormal physiological states early provided strong evidence of vitamin K deficiency. For example, rats (112) and dogs (114) were known to exhibit loss of blood coagulability and a low prothrombin level when deprived of bile by a fistula. As a sequel to their work on the relation of bile to the absorption of the fat-soluble vitamins, Greaves and Schmidt made the observation that the low prothrombin of bile-fistula rats could be corrected by oral administration of vitamin K, but not efficiently unless the vitamin was given with bile or bile salts (112). Quick, in a résumé of the status of vitamin K, independently suggested these same relations between prothrombin, bile and vitamin K (149). Working with bile-fistula dogs and using a different quantitative procedure for prothrombin, Smith et al. (168) obtained essentially the same results as reported for the bile-fistula rat.

Ligation of the bile duct was sufficient to bring about a low prothrombin value in the blood of rats on a stock diet (112) and in chicks on a diet containing adequate vitamin K (63). It was also observed that feeding bile alone was capable of causing elevation of prothrombin level in these animals with ligated bile ducts probably through facilitated absorption of the vitamin in the diet or from bacteria in the bowel (109, 110, 112, 168). Bile alone has long been known to act against the bleeding tendencies frequently associated with human obstructive jaundice (175) but the explanation of this antihemorrhagic action has been

lacking. It is of interest to note that bile contains no appreciable amount of vitamin K (9, 110).

The bile component principally concerned in the absorption and transport of fat-soluble vitamin K from the digestive tract is undoubtedly deoxycholic acid (165). The molecular compound of vitamin K with deoxycholic acid (vitamin K-choleic acid, 11) was effective upon oral administration to bile-fistula rats (55). A crude vitamin K-deoxycholic acid preparation in aqueous solution was found effective by subcutaneous injection into deficient chicks, whereas an emulsion of the crude vitamin was effective by intramuscular but not by subcutaneous injection (69). It is pertinent to add a recent report that dioctyl sodium sulfosuccinate, a highly "surface-active" compound, can facilitate the absorption of a fat-soluble antihemorrhagic substance, methyl naphthoquinone (131).

*Hemorrhagic tendencies in obstructive jaundice.* From this realization of the rôle of bile and the original observations by Quick, Stanley-Brown and Bancroft of low prothrombin levels in certain cases of obstructive jaundice (159), Smith and co-workers (47, 191) and Snell et al. (175) proceeded successfully to overcome the bleeding defect in obstructive jaundice by administration of crude vitamin K with bile or bile salts. Dam and Glavind (63, 64, 65) early employed intramuscular injection of vitamin K concentrates dissolved in oil, thus dispensing with the use of bile salts which some jaundiced patients can not tolerate. Further confirmatory reports have described the clinical use of crude, purified, or synthetic sources of antihemorrhagic activity (2, 29, 49, 50, 51, 52, 127, 145, 147, 160, 161, 164, 169, 170, 176, 177, 191) administered by various routes.

Synthetic substitutes for vitamin K were found effective by intraperitoneal injection in bile-fistula rats, as absorption can proceed by this route without the agency of bile (105). Recently, the tendency has been to employ the water-soluble substitutes which can be given intravenously (2, 51, 52, 162). Highly water-soluble vitamin K substitutes now available appear to be absorbed directly from the intestine independently of bile (192).

It is generally agreed that obstructive jaundice is fully analogous, both in the fundamental nature of the bleeding tendencies and in the mechanism of the cure wrought by vitamin K and bile, to the bleeding disease of experimental animals in which drainage of bile to the intestine is prevented. The aforementioned developments bridged the gap between the dietary K-avitaminosis of the chicken and the bleeding tend-

ency of the jaundiced patient. Other clinical applications of vitamin K soon followed.

*Hemorrhagic disease of the new-born.* The hemorrhagic disease of the infant is now recognized as a type of alimentary vitamin K deficiency characterized by a dangerously low prothrombin level during the first few days of life (46, 123, 144, 146, 151, 156, 158). Although recovery may be spontaneous, perhaps through the establishment of an intestinal flora after ingestion of food (156, 157, 158), treatment with vitamin K orally effects a prompt recovery (75, 76, 115, 144, 148, 188, 189). Prothrombin in the blood of the infant can also be increased by giving vitamin K to the mother previous to delivery (115, 167). For a more detailed review of the hemorrhagic disease of the infant the reader is referred to papers by Quick and Grossman (156) and Grossman (113).

*Hemorrhagic disease accompanying liver damage.* Another type of hemorrhagic disease in which vitamin K appears to be concerned is that which sometimes follows severe liver damage. Schmidt (165) and Snell (172) called attention to liver function and liver damage as possible major factors in the metabolism of vitamin K and the synthesis of prothrombin. This idea has been given further emphasis (50, 111, 153, 154, 164, 169, 174, 190).

Trauma produced by operation and massage of the liver results in a marked loss of blood prothrombin which may last for several days (130). Extirpation of the liver also causes a striking decrease in prothrombin (30, 190) which is not corrected by the level of vitamin K with bile that suffices for the cure of biliary fistula animals (30). The hypoprothrombinemia which develops after liver injury from chronic chloroform intoxication is apparently not influenced by vitamin K (48). Cases of primary liver disease such as atrophy, hepatitis, cirrhosis and carcinoma frequently involve hypoprothrombinemia. Such cases have actually shown slow or no response to vitamin K given intravenously, intramuscularly, or orally (2, 29, 52, 147, 164). In many such cases, however, extensive liver damage apparently may exist without great loss of prothrombin, the formation of which seems to be one of the last liver functions to be impaired (50, 52, 63, 140, 173).

*Hemorrhagic disease from other causes.* Rarer conditions in which vitamin K absorption is hindered and prothrombin level may consequently fall include non-tropical sprue (2, 54, 84), biliary fistula (87, 170), ulcerative colitis and other abnormalities of the intestinal surface (54). Such conditions have been successfully treated with vitamin K preparations. Chicks receiving an adequate level of vitamin K in

the diet can quickly be rendered deficient in prothrombin by an addition to the diet of activated carbon, which firmly adsorbs the vitamin.[1] Feeding high levels of mineral oil in the diet causes a lowered blood prothrombin apparently by interference with absorption; this effect can be corrected by injection of vitamin K (82).

Treatment of hemorrhagic retinitis with vitamin K has yielded favorable results (141). The bleeding disease of animals fed spoiled sweet clover hay appears to resemble vitamin K deficiency in that there is a decrease in prothrombin which can be remedied by feeding good alfalfa hay (149). On this latter point there is, however, lack of agreement (171).

*Hemophilia.* The clotting defect in hemophilia quite clearly does not involve prothrombin deficiency (45, 50, 63, 65, 164, 159), hence it does not respond to vitamin K administration (53, 63, 164). The nature of the defect in hemophilia appears to be a lack or inavailability of blood agents which cause the change of prothrombin to thrombin, i.e., thromboplastin (45, 159).

*Diagnostic tests.* Diagnostic and "bedside" tests for prothrombin deficiency have been proposed most of which are based on the principle of eliminating thromboplastin variations by providing a clotting agent in excess and comparing the clotting time of the tested blood with that of normal blood under the same conditions (146, 152, 193, 194), or of comparing the thromboplastin concentrations required to produce a standard clotting time in the tested and normal blood specimens (63).

*Mechanism of vitamin K action.* Very little is known about the manner in which vitamin K promotes the formation of prothrombin. That the location of this action is in the liver seems to be well established.

An early suggestion that vitamin K may exist as a prosthetic group on the prothrombin molecule (74) was not supported by attempts to detect vitamin K in large quantities of the prothrombin fraction of normal chicken blood (69). Dried beef blood fed at a level of 10 per cent to deficient chicks also showed no activity.[2] It seems unlikely that the vitamin occurs in blood except during transport.

An interesting suggestion has been made that the antihemorrhagic naphthoquinones can oxidize the —SH groups of certain proteins, such as prothrombin, forming —SS— linkages between protein molecules (40). That the action of the vitamin is not simple, however, is indicated by the failure of the vitamin in contact with prothrombin deficient

[1] Unpublished data of the author.

[2] Unpublished data of the author.

chick blood to accelerate clotting (69, 74). Even the water-soluble forms, such as vitamin K-choleic acid, phthiocol, methyl naphthoquinone and the diphosphoric acid ester of methyl naphthohydroquinone, have no direct effect on deficient blood.[2]

Due to recent publicity given oxalic acid as a blood clotting agent, investigations were made of the effect of oxalic acid in vitamin K-deficient chicks and in heparinized rabbits. The oxalic acid had no detectable effect (106). Similar results were obtained in the case of vitamin K-deficient chicks after intraperitoneal injection of sodium oxalate.[2]

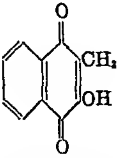
Studies of the lethal dosage of vitamin $K_1$ and two of its substitutes, phthiocol and methyl naphthoquinone, have indicated some toxic effects with the two substitutes at levels much above normal therapeutic levels (142). In clinical usage these compounds have given no evidence of toxic after-effects (2, 51).

The author has omitted many brief or confirmatory reports of a clinical nature. For a more complete account of the therapeutic use of vitamin K, the reader is referred to recent clinical reviews by Quick (150, 153, 154), Snell (172), Snell and Butt (173, 174), Dam (61), and Ferguson (86).

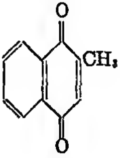
Biochemistry. Procedures which aided in the purification of the vitamin as found in alfalfa were published from time to time by Dam and co-workers (70, 72) and by Almquist and co-workers (3, 4, 6, 7, 8, 11, 125). These methods involved extraction with fat solvents, precipitation of impurities from solution in various solvents, adsorption and molecular distillation. The progress on the structure of vitamin K may be most coherently presented from the standpoint of the different groups of workers that engaged in the work.

*The European group.* Dam, Karrer, et al. announced the isolation of vitamin K in a highly purified form (62). The final preparation retained a constant composition and activity after repeated chromatographic adsorption. It contained carbon—82.2, hydrogen—10.7 per cent, and 2 atoms of oxygen per molecule. Absorption maxima were noted at 248, 261, 270 and 328 mμ. The extinction coefficient, $E_{1\,cm}^{1\,per\,cent}$, for the wave length 248 was 280. A characteristic color reaction of the vitamin in sodium ethylate solution was described during which an intense blue color later turning to reddish-brown developed (62, 119). Because of the fact that the product was an oil, dependable criteria for absolute purity could not be provided. Work continued in Karrer's laboratory (120) indicated a provisional formula of

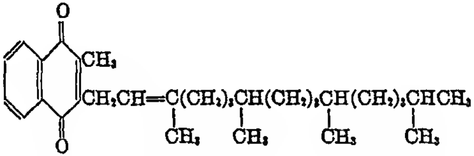
$C_{30-32}H_{46-48-50}O_2$, a molecular weight of 445–50, and that the vitamin was probably a relatively stable quinone. The name "α-phylloquinone" was suggested by Dam (121) but the information presented by the



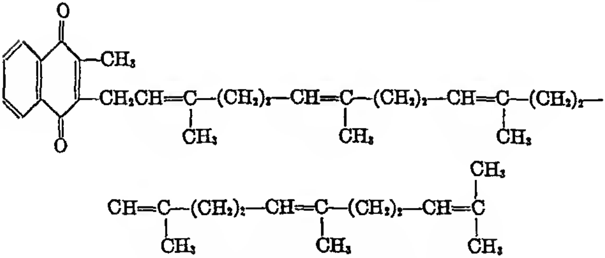
PHTHIOCOL
2-methyl-3-hydroxy-1,4-naphthoquinone



2-methyl-1,4-naphthoquinone



2-methyl-3-phytyl-1,4-naphthoquinone (vitamin $K_1$)



Vitamin $K_2$ (proposed structure)

European workers at this stage could hardly be regarded as warranting the selection of a name based on structure. Details of the final method of isolation were ultimately published (121). Synthesis of a 2-dihy-

drophytyl-1,4-naphthoquinone with physical and chemical properties similar to those of the "α-phylloquinone," except for failure of the blue color test, was also announced (122).

*The St. Louis group.* Independently of the European group, Doisy and co-workers at St. Louis (McKee et al., 138) had obtained two final products, one from alfalfa ($K_1$) and one from putrified fish meal ($K_2$). The $K_1$ extinction coefficient, $E_{1\,\text{cm}}^{1\,\text{per cent}}$, for the band at 248 mμ was 385, suggesting that the product was purer than the one described by Dam, Karrer et al. $K_1$ was a yellow oil while $K_2$ was a yellow crystalline solid. Nitrogen, sulphur, phosphorus and halogens were absent from both preparations. Neither was optically active. $K_1$ contained carbon—82.76, 82.54, hydrogen—10.65, 10.66. Its molecular weight was determined as 443, 464, and probable formula $C_{32}H_{48-50}O_2$. From physical data the suggestion was made that vitamin K contained a quinoid structure (138).

While there was some controversy over the relative purity of these preparations from the two laboratories, based primarily on the differences in extinction coefficients (42, 85, 120, 138) the biological activity of preparations from the two sources was found to be essentially identical in simultaneous quantitative assays by a third laboratory (17, 20).

Crystalline diacetates of $K_1$ and $K_2$ were next prepared by reductive acetylation and the vitamins were regenerated without change in potency (42). Ozonolysis of the $K_1$ diacetate led to the identification of one fragment of the vitamin as 2,6,10-trimethylpentadecanone-14, which can also be obtained from phytol, and another fragment tentatively identified as 2-ethyl-1,4-naphthoquinone-3-acetic acid (133). More drastic oxidation by chromic acid yielded phthalic acid (133). The quinone acid was finally indentified with 2-methyl-1,4-naphthoquinone-3-acetic acid (41). This work suggested that vitamin $K_1$ is 2-methyl-3-phytyl-1,4-naphthoquinone, since the only places left for substituents were positions 2 and 3 on the quinone ring. Synthesis of this compound was effected through reaction of phytyl bromide with monosodium-2-methyl-1,4-naphthohydroquinone and purification by chromatographic adsorption and molecular distillation (Binkley et al., 41). The diacetate of the synthetic product was identified with that of the natural vitamin $K_1$ (41, 135). This work was later described in more detail (134) and the procedure for isolation of the natural vitamin $K_1$ subsequently given (43).

The procedure for the preparation and isolation of vitamin $K_2$ by the St. Louis group was also described (139). In a continuation of their classical degradation studies on these vitamins, it was found that the

same quinone acid and phthalic acid could be obtained from $K_2$ (44). This vitamin was evidently analogous to $K_1$, but with a much longer and more unsaturated side chain at position 3. The correct empirical formula was given as $C_{41}H_{56}O_2$ and a structural formula was proposed (44). The ultraviolet absorption of these pure vitamins was studied in detail (85). Reviews of the work of the St. Louis group have been published (79, 80).

*The Harvard group.* Fieser and associates at Harvard became interested in the synthesis of vitamin $K_1$, after considerable information on its chemical nature had been released showing that the vitamin was probably a naphthoquinone. In a series of preliminary papers, they described steps leading to the synthesis of 2-methyl-3-phytyl-1,4-naphthoquinone which they correctly surmised to be identical with vitamin $K_1$ (99, 100, 101, 104). They also suggested, incorrectly, that vitamin $K_2$ might be 2,3-difarnesyl-1,4-naphthoquinone (95, 99). Synthesis of 2-methyl-3-phytyl-1,4-naphthoquinone was then reported by Fieser (92, 94) and similarity shown between this compound and natural vitamin $K_1$ in respect to absorption spectrum, color reaction, antihemorrhagic activity, melting point of the diacetate, and elementary composition (92, 93). Convenient methods for the synthesis of several of the more important antihemorrhagic compounds were reported (96). The work of this group has also been reviewed (95).

*The California group.* The work of Almquist and associates followed yet another course. It appeared from the effect of specific group reagents on the potency of active concentrates that the vitamin was complex, unsaturated, probably aromatic in part, and lacking in alcoholic or phenolic groups (6, 126). Reagents for ketone groups also failed to affect activity (8, 70, 126). The vitamin apparently had oily properties at room temperature and could be distilled under greatly reduced pressure (4, 8, 70). The purest concentrates contained no sulphur, phosphorus or nitrogen (6, 8). The vitamin was readily destroyed by exposure to ultraviolet light (6, 8, see also 132) and by basic substances (4, 8, 72).

Studies of the alkaline degradation products of the vitamin were pursued with the view of isolating some derivative to furnish a clue on structure. There was obtained an oily, pigmented, acidic derivative with carbon and hydrogen analyses and molecular weight suggesting a provisional formula of $C_{31}H_{50}O_4$ (18). It was apparent that this derivative was greater than the vitamin $K_1$ by approximately two molecules of water.

As a free acid the derived pigment was clear yellow in color, in al-

kaline solution it was reddish-brown. It became evident that the pigment was an end-stage of a color reaction for vitamin $K_1$ reported meanwhile by Dam, Karrer et al. (62). On the basis of the reddish-brown terminal color, close correlation between color intensity and activity was found in a variety of preparations (12).

Among a number of bacteria tested, the tuberculosis organism had been found to possess appreciable activity (23). In 1933–34, the principal pigment of this organism had been isolated and synthesized by Anderson and co-workers at Yale. The pigment, phthiocol, was 2-methyl-3-hydroxy-1,4-naphthoquinone. The attention of the California workers was drawn to this compound because it was a quinone pigment like vitamin K, because of its presence in a natural active product, because it exhibited color changes similar to those of the oily pigment derived from vitamin K by alkaline hydrolysis and because its absorption curve compared closely with that already given for preparations of natural vitamin $K_1$ (14, 138). A sample of synthetic phthiocol, obtained from Professor Anderson, was tested with chicks and found to be active in restoring normal blood clotting time (13, 14).

Although more potent synthetic compounds were later discovered, phthiocol is of historic importance in that it was the first pure, natural, antihemorrhagic compound of known structure to be announced and in that its activity confirmed suggestions of a quinoid character of the vitamin, indicating more specifically a naphthoquinoid structure. Phthiocol was probably the first synthetic form of vitamin K to be employed with success in human cases (51, 169).

2-methyl-1,4-naphthoquinone was found to be more active than phthiocol (14, 15) and, subsequently, by oral administration several times as active as natural vitamin $K_1$ (17), after low and erratic values obtained by administration of the methyl naphthoquinone in the diet were found due to some cause of loss not formerly encountered. It seemed likely that this quinone could represent the active nucleus of the vitamin and that the remainder of the vitamin not yet accounted for might logically be a phytyl side chain, because of the presence of phytol in alfalfa and because a compound of the methyl naphthoquinone and phytol could be expected to conform to the reported physical and chemical properties of vitamin $K_1$. Since the vitamin lacked an alcoholic group and was not split by mild alkaline hydrolysis, the phytol was evidently combined through a carbon-carbon bond as a phytyl group. Natural vitamin $K_1$, the alkaline derivative of the vitamin, and pure phytol, but not the methyl naphthoquinone, were also ob-

served to exhibit the same characteristic fluorescence in ultra-violet light (15). (Some synthetic samples of vitamin $K_1$ have not yielded this fluorescence, which may have been due entirely to free phytol present in traces.[3])

The compound 2-methyl-3-phytyl-1,4-naphthoquinone was synthesized from methyl naphthoquinone and phytol, through the hydroquinone and phytyl bromide, and purified by repeated molecular distillation and crystallization at low temperatures. The product was found to resemble natural vitamin $K_1$ in elementary composition, color reaction and potency (15, 124).

The first reports of the synthesis of this compound by three laboratories in this country appeared in the same issue of the same journal (15, 92, 41). It may be pointed out that the methods for synthesis did not preclude the possibility of extensive coupling at positions 5, 6, 7 and 8, although position 3 would be expected to be the most reactive. Such isomers, as well as some cyclized by-products, may well be present in synthetic preparations. It was the isolation by the St. Louis group of 2-methyl-1,4-naphthoquinone-3-acetic acid from the oxidation products of both natural and synthetic preparations which conclusively proved their identity (135).

*Vitamin K substitutes.* In contrast to most other vitamins, synthetic vitamin $K_1$ did not prove to be the ultimate goal but only a way-station. This was due to the rather exceptional fact that a portion of the vitamin was more active than the whole natural vitamin. Immediately after the report on phthiocol (13), a number of accounts appeared of activity in other naphthoquinones, mostly based on rapid 6 to 18 hour tests (35, 97, 98, 179). Ansbacher and Fernholz confirmed the activity of phthiocol and reported further that 2-methyl-1,4-naphthoquinone was practically as active as vitamin $K_1$ (35). This claim was supported by others (178, 187) after earlier reports of lower activity. Further assays have revealed that this compound has an even higher relative activity, between 2 and 4 times that of vitamin $K_1$ (17, 19, 20, 33, 78, 83, 88, 181).

Since 2-methyl-1,4-naphthoquinone can be obtained readily in a high state of purity and its physical properties are accurately known, it has been proposed as a basic standard of potency (178). It is reasonably stable to light, although the light condensation dimer is probably inactive (20). Either this compound or 2-methyl-1,4-naphthohydroquinone diacetate, which is probably more stable (85) but only one half as active (20, 37, 179), should serve very well for a standard. The di-

[3] Unpublished data of the author.

acetate appears to suffer no loss of activity, like that found with the free quinone, when given in the diet as compared to daily oral dosage.[4] The reproducibility of these compounds would add the advantage that an international standard sample need not be preserved.

2-methyl-1,4-naphthoquinone is sufficiently water-soluble at room temperature, 0.1 mgm. per ml., (0.13 mgm. per ml.[4]) to allow its use intravenously (38). One milligram daily has been suggested as an adequate clinical dose (88). The methyl naphthohydroquinone is more water-soluble but equally as active (20, 38, 178), probably because it is readily subject to oxidation on contact with air.

The compound, 4-amino-2-methyl-1-naphthol hydrochloride, is quite water-soluble and approximately as potent as vitamin $K_1$ (81). It has also been variously reported to be about one-half as potent (20) and equally as potent (162) as methyl naphthoquinone into which it is probably metabolized (80). 4-amino-3-methyl-1-naphthol hydrochloride is similarly active (162).

A large number of compounds capable of conversion to 2-methyl-1,4-naphthoquinone by easy stages during metabolism probably owe their activity to such conversion. Among these compounds are 1-hydroxy-2-methyl and 1-hydroxy-3-methyl naphthalene, 1-amino-2-methyl naphthalene, 3-methyl-1-tetralone, and 2-methyl-1-tetralone (186). In this same report it was shown that 1-methyl-2-hydroxy, 2-methyl-3-hydroxy, and 1-methyl-4-hydroxy naphthalenes were inactive at high levels. The latter compounds are not convertible to 2-methyl-1,4-naphthoquinone. The comparatively low potencies of compounds like 2-methyl-3-hydroxy, 2,3-dimethyl, and 2-ethyl-1,4-naphthoquinone are thus explainable on the basis that removal of the hydroxy or the second methyl group or conversion of the ethyl to a methyl group would not be an easy metabolic process.

A series of di-esters of the methyl naphthohydroquinone were prepared and found potent, probably because of hydrolytic regeneration of the methyl naphthoquinone (37). An apparent exception was encountered in the case of the quite active 2-methyl-1,4-dimethoxynaphthalene, which presumably could not be readily hydrolyzed (37), but it has been pointed out that this compound could be oxidized to the methyl naphthoquinone (186).

Comparative assays of vitamins $K_1$ and $K_2$ have indicated that the potencies of these compounds are in the ratio of 1.25 to 1, which is also the ratio of the percentage of methyl naphthoquinone combined in each

[4] Unpublished data of the author.

(20). Assays by an 18 hour procedure indicated a higher potency ratio of 1.52 (179, 182). It is obvious that the long side chains in these vitamins are not specific; in fact, the methyl naphthoquinone is more active without the side chains (17, 20) which are probably removed in metabolism.

Numerous other naphthoquinones have been found active to some degree (20, 89, 91, 97, 103, 179, 187). Following phthiocol, several naturally occurring 3-hydroxy naphthoquinones, having a longer side chain in place of the methyl group, i.e., lapachol, hydrolapachol and lomatiol, were reported to show some activity (97), but another report at the same time indicated no activity (14) and was later confirmed (99). Various derivatives of phthiocol were assayed but no exceptionally high potency found (15). The list of antihemorrhagic substances is now too large to be given in detail. Many of these compounds do not possess practical value.

The most surprising development in recent studies of simple, synthetic vitamin K substitutes is the report of Foster et al. that 2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester is even more active on the molecular basis than the methyl naphthoquinone (108). This fact encouraged the suggestion that the metabolism of methyl naphthoquinone might involve phosphorylation as an early step (108), but, of course, it could also mean that the diphosphoric acid ester is more efficiently absorbed than any other antihemorrhagic compound, and that after absorption the quinone is readily regenerated. Either assumption could explain the lower activity of the disulphuric acid ester (102, 162) from which the quinone would not be so readily recovered. Another report has maintained that the activity of the diphosphoric acid ester is but 1/20 that of the methyl naphthoquinone (38). The high activity of the diphosphoric acid ester has been amply confirmed, however, and appears to be approximately 50 per cent greater on the molecular basis than that of the methyl naphthoquinone (20, 107). Pharmacological studies on this compound indicate a wide margin of safety and no adverse effects in therapeutic doses (107, 108).

Claims for an additional form of vitamin K in alfalfa have been made. These are based on the separation from alfalfa extracts of a fraction which did not give the typical strong color reaction in sodium ethylate, yet manifested considerable activity (39, 90). It was nearly colorless and was later reported to be more active than synthetic 2-methyl-3-phytyl-1,4-naphthoquinone in a 6 hour test, but not in an 18 hour test (39). 2-methyl-3-"isophytyl"-1,4-naphthoquinone was prepared

and found comparable to synthetic vitamin $K_1$ in activity (39). It is interesting, in this connection, that vitamin $K_1$ can be converted to a 2,3-oxido derivative, a nearly colorless oil, which is as active as vitamin $K_1$ but which gives no Dam-Karrer color reaction, and resembles in these respects the second form of vitamin K reported to be present in alfalfa (103).

The activity of these nearly colorless products (and of the colorless reduced forms and diesters of the vitamins) may explain the potency found in some earlier, semi-refined, preparations which had very little or no color (7, 8, 129, 183) and in which the vitamin may have been present in a mixed crystal, or perhaps adsorbed on an inert crystalline medium.

TABLE 2

*Comparative activities of the more important antihemorrhagic compounds based on recent chick 5-day assays and expressed in 2-methyl-1,4-naphthoquinone units per milligram and per micromol*

| | UNITS PER MILLIGRAM | UNITS PER MICROMOL |
|---|---|---|
| 2-methyl-1,4-naphthoquinone | 1,000 | 172 |
| 2-methyl-1,4-naphthohydroquinone | 930 | 162 |
| 2-methyl-1,4-naphthohydroquinone diacetate | 450 | 116 |
| 2-methyl-4-amino-1-naphthol hydrochloride | 470 | 99 |
| 2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester (tetra sodium salt + 6 molecules water) | 490 | 260 |
| 2-methyl-3-phytyl-1,4-naphthoquinone (vitamin $K_1$): | | |
| Natural | 300 | 136 |
| Natural, dihydro diacetate | 100 | 54 |
| Synthetic | 290 | 131 |
| 2-methyl-3-(?)-1,4-naphthoquinone (vitamin $K_2$) | 240 | 139 |

Only one outstanding example of activity is definitely known where conversion to methyl naphthoquinone does not seem possible. This is in the case of 2,5-dimethyl-benzoquinone (36) which has on both sides of its structure the same configuration as present along the 1, 2, 3, 4 positions of 2-methyl-1,4-naphthoquinone. Antihemorrhagic activity of this benzoquinone, although very slight, was detectable and has been confirmed (approximately 1/10,000 that of methyl naphthoquinone[5]). A degradation product of vitamin E, $\alpha$-tocopherylquinone, apparently has some slight antihemorrhagic activity (128). Activity comparable to that of 2,5-dimethyl-benzoquinone has also been found in perezone, which is probably a benzoquinone.[5] It seems possible,

[5] Unpublished data of the author.

therefore, that certain compounds may act feebly in their original form or through some general property of the quinones.

Vitamin K, like the other fat-soluble vitamins, has now expanded into an extensive group of biologically active compounds. It would not be surprising if additional, naturally occurring or synthetic forms should be discovered. A comparison of the activities of some of the more important known compounds is given in table 2.

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# CHANGING CONCEPTS OF THE CHEMISTRY OF MUSCULAR CONTRACTION

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There is no chapter in modern physiology which presents a more interesting development than the one dealing with the nature of the chemical reactions which supply the energy for contraction of striated muscle. New discoveries, each calling for new orientations, have come in such rapid succession as to bewilder and confuse the observer. In the eight years since Hill (39) described the "revolution in muscle physiology" which necessitated a complete revaluation of earlier data, there have been so many new findings that the prediction made therein: "in a few years further discoveries will lead to further drastic change" has been amply justified.

Many of the interpretations in this field have been influenced by a conception of the Pasteur reaction that involves a significant departure from Pasteur's statement of the relation between respiration and fermentation. In the authorized translation of Pasteur's *Etudes sur la Bière* (76) the following occurs:

> . . . fermentation is a chemical action . . . that takes place when these cells, ceasing to have the power of freely consuming the materials of their nutrition by respiratory processes . . . continue to live by utilizing oxygenated matters which . . . produce heat by their decomposition. The character of ferment thus presents itself to us, not as peculiar to any particular being or to any particular organ, but as a general property of the living cell. The character is always ready to manifest itself, and, in reality, does manifest itself, as soon as life ceases to perform its functions under the influence of free oxygen, or without a quantity of that gas sufficient for all the acts of nutrition (p. 114–115).

The modern interpretation of this statement is that fermentation is the fundamental metabolic process, and that respiration is merely a means of decreasing the excessive carbohydrate breakdown inherent in such an inefficient mechanism.

The observations of Hill (36) on the heat relations in muscular contraction were interpreted in these terms. Hill found that the heat liberated during the contraction was the same, per unit of tension developed, whether the muscle were in an atmosphere of oxygen or nitrogen, and drew the conclusion that the chemical reactions which supplied the energy for the contraction were non-oxidative in the presence of oxygen as they must be in its absence. In oxygen, the muscle showed a liberation of heat in the period following the completion of the mechanical event. This was interpreted as evidence of an oxidative recovery process. Since the heat liberation during the recovery period was only a fraction of that which would result from the oxidation of all the lactic acid formed during contraction, the major part must be resynthesized to the glycogen from which it was formed.

Meyerhof (62) then found that the extra oxygen consumed during the recovery period was sufficient to oxidize only one-fifth of the lactic acid which disappeared. This finding led to the concept that the steady state was maintained by continuous anaerobic breakdown and oxidative resynthesis of carbohydrate, with the same amount of lactic acid formed in unit time in the presence of oxygen as in its absence. In oxygen, the major portion was resynthesized to glycogen by the energy liberated in the oxidation of the smaller portion. Thus did the "Pasteur reaction" become the "Meyerhof cycle."

Hartree and Hill (32) then showed that the heat relations were in accord with Meyerhof's chemical data, in indicating that four molecules of lactic acid were resynthesized to glycogen for each one oxidized. They were able to draw up a thermal balance sheet which accounted for all the heat liberated in contraction and recovery in terms of formation and neutralization of lactic acid in contraction, and recovery by the Meyerhof cycle. They found no residual heat which could be attributed to other chemical processes.

This rather simple formulation was disturbed when Embden *et al.* (18) found that under anaerobic conditions the formation of lactic acid continued into the period *after* tetanus. Meyerhof and Lohmann (64) regarded this "delayed" lactic acid formation as an artefact due to "overstimulation." Hill (37) was unable to find any heat production following a series of twitches, and therefore concluded that no lactic acid formation or other exothermic process took place after the contraction was over.

The discovery of phosphocreatine in muscle by Fiske and Subbarow (25) and the finding by Eggleton and Eggleton (17) that it underwent

hydrolysis in contraction and resynthesis in oxidative recovery, initiated a series of investigations which culminated in the "revolution" described by Hill. In addition to the hydrolytic reaction, it was shown (16) that there was reaction between glycogen and phosphocreatine to form the hexosemonophosphate which Embden and Zimmerman (19) postulated as an intermediate in the formation of lactic acid. There was also found (72) resynthesis of part of the phosphocreatine in the post-tetanus period under anaerobic conditions.

Fiske and Subbarow (26) demonstrated that phosphocreatine is a much stronger acid than the phosphoric acid it yields on hydrolysis; the $pK'_2$ values are 4.6 and 7.0, respectively. They suggested that the function of the hydrolytic reaction in contraction was to supply alkali to buffer the lactic acid formed.

Attention was diverted from this important suggestion by the discovery made by Lundsgaard (54) that frog muscles poisoned with iodoacetic acid could contract anaerobically without the formation of lactic acid. After a number of apparently normal contractions, the muscles passed into rigor and contracture. In the period of normal contractions, the principal reaction taking place was the formation of hexosemonophosphate from glycogen and phosphocreatine; there was also some hydrolysis of phosphocreatine. Lundsgaard used these observations, together with those of Embden (18) on post-tetanus formation of lactic acid, and of Nachmansohn (72) on anaerobic resynthesis of phosphocreatine, as the basis for the hypothesis that the normal source of the energy for contraction is the hydrolysis of phosphocreatine, and that the formation of lactic acid *after* contraction brought about recovery by supplying energy for the resynthesis of phosphocreatine. Thus the first stage in recovery became an anaerobic process, and oxidative reactions were pushed further into the background.

In the normal muscle, most of the phosphocreatine which disappears in contraction undergoes hydrolysis; in the poisoned one, the principal reaction is the conversion to hexosemonophosphate. Lundsgaard at first considered that the formation of hexosemonophosphate from inorganic phosphate and glycogen supplied the energy for the resynthesis of some phosphocreatine, but soon found this untenable. The two pathways of phosphocreatine breakdown were then regarded as being equivalent in function. Meyerhof, Lundsgaard, and Blaschko (67) found that the heat of hydrolysis of phosphocreatine was sufficient to meet the energy requirements of contraction; Meyerhof and Schulz (70) found that the formation of lactic acid after tetanus was not an

artefact due to "over-stimulation"; and Hartree and Hill (33) succeeded in demonstrating the existence of post-tetanus anaerobic heat. This series of events was the occasion for Hill (39) to describe the "revolution" in muscle physiology. To quote from this paper: "Thus it is reasonable to regard the *whole* of the lactic acid set free as being concerned with the restoration of phosphagen broken down during activity. . . ." There remained to be answered the question of why the anaerobic resynthesis was incomplete, for Nachmansohn (73) had shown that it was limited to some 30 per cent of the amount hydrolyzed during the tetanus. There were also some discrepancies in the time relations, for the anaerobic resynthesis which did take place was complete in less than a minute, whereas lactic acid formation continued for more than 5 minutes after the end of the tetanus (50) and delayed anaerobic heat continued to be liberated even longer than this (10).

This formulation had scarcely been completed when another modification became necessary. Meyerhof and Lohmann (65) found that at pH 8.0 to 9.0, the addition of adenosine triphosphate to muscle extract containing polysaccharide led to a reaction in which phosphocreatine and adenylic acid were formed. They postulated that this reaction was the immediate source of the energy for the resynthesis of phosphocreatine, and that the formation of lactic acid at a later stage in anaerobic recovery supplied the energy for the resynthesis of adenosine triphosphate. However, Lundsgaard (57) found that there was no breakdown of adenosine triphosphate in the iodoacetate-poisoned muscle giving single twitches, until after the onset of rigor. He also noted that the poisoned muscle showed additional breakdown of phosphocreatine after tetanus, instead of the resynthesis shown by normal muscle.

Lohmann (53) then found that the phosphate interchange was reversible; at pH 7.0, adenylic acid reacted with phosphocreatine to form adenosine triphosphate and creatine. The hypothesis now became that the initial reaction in contraction was the breakdown of adenosine triphosphate, and that the recovery process began with the resynthesis of this substance by phosphocreatine. At a later stage in anaerobic recovery, the phosphocreatine was resynthesized by the energy of formation of lactic acid, and the final recovery was accomplished by oxidative reactions. In the further evolution of the anaerobic contraction theory, phosphocreatine has been relegated to a still less important position, although it is the one phosphorus compound in muscle which is most likely to undergo change in contraction.

In the experiments referred to above, frog muscle or muscle extract was used. The development by Davenport (15) of the technique of freezing the muscle *in situ* with a mixture of powdered solid carbon dioxide and a volatile liquid, made it feasible to study contraction of mammalian muscle with normal circulation. Application of this technique to the study of the changes produced when muscles of rabbits were subjected to tetani of varying durations, showed that in the first few seconds of contraction there was no hydrolysis of phosphocreatine, but large amounts of lactic acid and hexosemonophosphate were formed (85). The phosphate groups for hexosemonophosphate formation were supplied by phosphocreatine. With longer stimulation, phosphocreatine hydrolysis did appear, and the rates of accumulation of lactic acid and hexosemonophosphate fell off rapidly. When the stimulation period was sufficiently prolonged, there were decreases in the amounts of lactic acid and hexosemonophosphate present, and resynthesis of phosphocreatine was noted. It was difficult to reconcile these findings with the Lundsgaard hypothesis, for several reasons. In the initial period of contraction there was lactic acid formation without phosphocreatine hydrolysis, whereas the reverse situation was to be expected, on account of the postulated lag in anaerobic recovery. Also, the association of phosphocreatine hydrolysis with lactic acid formation gave a relation opposite to the requirements of this hypothesis. Finally, the rapid decrease in the rate of accumulation of anaerobic metabolites did not seem compatible with the idea that contraction is anaerobic. An alternative theory was therefore presented (85): that the fundamental chemical reactions by which the energy for muscular contraction is supplied are oxidative, and that anaerobic reactions are used only when the supply of oxygen is inadequate. The principal anaerobic reaction is the formation of lactic acid from glycogen; whenever this mechanism becomes insufficient, the formation of hexosemonophosphate from glycogen and phosphocreatine is used as a supplementary source of energy. Implicit in this is the concept that hexosemonophosphate is not an intermediate in the formation of lactic acid. The primary function of phosphocreatine hydrolysis is that suggested by Fiske and Subbarow (26), to supply base to neutralize the lactic acid formed in contraction under deficient oxygen supply. Insofar as it is used to form hexosemonophosphate, it serves as a source of energy, but this is limited to the supplementary rôle, and to anaerobic conditions.

Experiments were next made with rats, because Krogh (49) had found that the muscles of small animals contain a larger number of

capillaries per unit cross-section area than is the case with larger species. On this basis, it was possible that the oxygen supply might become adequate more rapidly after the onset of contraction than in the larger animal, and thus the cessation of formation of anaerobic metabolites take place much sooner. The results of the experiments (86) were in accord with this assumption. The accumulation of lactic acid and hexosemonophosphate ceased after only 15 seconds of tetanus in the rat muscles, whereas these processes continued into the second minute in the rabbit experiments. Also, the maximum lactic acid concentration found in the rat muscles was only one-third of the maximum found in the rabbits.

There was more phosphocreatine hydrolyzed in the rat muscles than in the rabbit muscles, relative to the lactic acid formation. The total alkali liberation from the reactions of phosphocreatine in the rat muscles was at least enough to buffer all the lactic acid formed. Most of the alkali was furnished by the hydrolysis of phosphocreatine, but the conversion of phosphocreatine P to hexosemonophosphate P also contributed some base, since this reaction forms an acid with a $pK'_2$ value of 6.1 (44) from one with $pK'_2$ value of 4.6 (26).

These calculations were made on the basis of pH 5.6 within the muscle fiber, since the studies of Rous (80) with indicator dyes favored this value, and it is also the pH at which the hydrolysis of phosphocreatine yields the maximum amount of buffer. However, Maurer (59) has shown that the type of indicator dye used by Rous penetrates only into those fibers which it injures. Fenn and Maurer (23) have used the carbon dioxide-bicarbonate and chloride relations of muscle and plasma to calculate the pH within the fiber, and arrive at the value of 6.9 for frog muscle. Hastings and Danielson (34) find pH 6.6 to 6.7 for mammalian muscle by the same method. Recalculation of the data on the stimulated rat muscles to this pH range shows that the alkali liberated by the phosphocreatine reactions is from 90 to 95 per cent of that needed to buffer all the lactic acid formed. A similar calculation from the data of Lundsgaard (56) on a short series of single twitches in frog muscle, on the basis of pH 6.9, shows that the alkali liberated was equivalent to the lactic acid formed. In anaerobiosis of frog muscle where the rate of lactic acid formation is accelerated by the presence of caffeine or dinitrophenol, there is similar equivalence between the alkali liberated by phosphocreatine hydrolysis and the lactic acid formed (13).

In seeking an explanation for the difference in the relative amounts

of phosphocreatine hydrolysis in the muscles of the two species, it was noted that the diet of the rats had an excess of acid-forming components, while that of the rabbits contained an excess of base. This could conceivably result in differences in the amounts of alkali present in the tissues in forms other than the salts of phosphocreatine. If Fiske's theory of phosphocreatine function be correct, then keeping the rabbits on an acid diet might prevent the accumulation of this other source of alkali and thereby lead to increased hydrolysis of phosphocreatine in anaerobic contraction. Parallel experiments were therefore carried out on two groups of young rabbits, one of which had been kept on an acid diet, the other on the alkaline one. It was found that in short periods of tetanus the same tension development, lactic acid formation, and hexosemonophosphate formation took place in the muscles of the two groups, but there was much more hydrolysis of phosphocreatine in the muscles of the "acid" rabbits (87). The total alkali liberation from phosphocreatine reactions in these rabbits was equivalent, at pH 6.6 to 6.7, to 75 to 95 per cent of the lactic acid formed, compared to about 40 per cent in the "alkaline" ones. It would appear that phosphocreatine hydrolysis does not take place until the other source of alkali is much reduced.

As a further test of the buffer theory of phosphocreatine function, the relations in the early part of the recovery period were studied. In cats and in rabbits it was found that the alkali removed by resynthesis of phosphocreatine from its hydrolysis products was practically equivalent, at pH 6.6 to 6.7, to the lactic acid lost by diffusion into the blood stream (88). Thus in contraction and in recovery, the relations found in mammalian muscle were those required by the buffer theory of phosphocreatine function, and opposite to those required by the hypothesis that the energy of formation of lactic acid is used to resynthesize phosphocreatine. It must be pointed out in this connection that Lundsgaard (56) found two moles of phosphocreatine *hydrolyzed* in a series of twitches, and two moles *resynthesized* after a tetanus, for each mole of lactic acid formed.

Additional evidence for the buffer theory of phosphocreatine hydrolysis is furnished by the observations of Lipmann and Meyerhof (52) that muscles, normal or iodoacetate-poisoned, exposed to carbon dioxide tensions greater than physiological, absorb carbon dioxide and hydrolyze phosphocreatine. The absorption is proportional to the concentration of carbon dioxide present and to the time of exposure. The effects are most marked in the absence of oxygen.

*Analysis of recovery rates in relation to contraction mechanisms.* These experiments on the recovery phase in mammalian muscle furnished a means of testing whether the assumption that lactic acid formation furnished the energy for resynthesis of phosphocreatine could account for the rate of energy production in anaerobic contraction. If this were to function as a recovery mechanism, then phosphocreatine resynthesis must take place with sufficient speed to meet the requirements of the physical situation. In 30 seconds of tetanus by a cat or rabbit gastrocnemius, the lactic acid formed amounts to 150 to 180 mgm. per cent, and in the first 60 seconds after such a tetanus, the resynthesis of phosphocreatine P amounts to 15 mgm. per cent (88). From the known heat of formation of lactic acid from glycogen, 269 cals. per gram (7), and heat of hydrolysis of phosphocreatine, 400 cals. per gram of P (67), it can be calculated that the assumption underlying the Lundsgaard hypothesis would require the resynthesis of phosphocreatine to take place at the rate of 100 to 130 mgm. per cent of P per minute, or more than 12 times the rate actually found. It must again be emphasized that this resynthesis in the recovering muscles is associated with the removal of lactic acid rather than its formation.

A similar calculation can be made for the anaerobic resynthesis of phosphocreatine in frog muscles. This proceeds at a rate not in excess of $\frac{1}{2}$ mgm. per cent of P per second (72). Lactic acid formation in anaerobic tetanus amounts to 4 mgm. per cent per second (24). From the heats of reaction it can be calculated that this rate of lactic acid formation is thermally equivalent to the resynthesis of 3 mgm. per cent of phosphocreatine P per second, or 6 times the maximum rate actually found. Thus the hypothesis that lactic acid formation in contraction serves to resynthesize phosphocreatine requires the assumption that recovery take place much more rapidly during contraction than it does afterward, and that the rate of recovery be greatest when the rate of doing work is greatest. But measurements of the heat production (10) show that the liberation of "recovery" heat is not accelerated by more intense activity. In mammalian muscle it has been found that neither formation of lactic acid nor resynthesis of phosphocreatine takes place under anaerobic conditions in the period following a tetanus (82). Application of the Lundsgaard hypothesis to this situation would require the assumptions that recovery is simultaneous with contraction and that the quantity of phosphocreatine that can be resynthesized anaerobically is limited only by the capacity of the muscle to form lactic acid. In the experiments referred to, the postulated

amount resynthesized would be greater than the quantity originally present.

An alternative explanation has been offered for the anaerobic resynthesis of phosphocreatine that has been observed in frog muscle (88), namely, that this is the means utilized by the muscle to remove alkali from the fiber to compensate for the loss of lactic acid by diffusion into the extracellular spaces. It is well known that diffusion of lactic acid from the muscle fiber is not accompanied by diffusion of base. Unless some compensatory removal of alkali took place within the fiber, the pH would rise considerably. The resynthesis of phosphocreatine from its hydrolysis products is the most efficient and the most readily available mechanism for compensating for this loss of acid. On this basis there is no difference between the resynthesis of phosphocreatine in mammalian muscle which accompanies the diffusion of lactic acid into the blood stream after a tetanus (88), and the resynthesis under anaerobic conditions in frog muscle.

*Position of oxidative mechanisms in contraction.* Within the past few years there have been some papers in which a more direct utilization of oxygen was indicated than that implied by the Lundsgaard theory. The interpretations in most of these have been made in terms of direct oxidative resynthesis of phosphocreatine instead of anaerobic resynthesis by lactic acid formation. This is the case in the studies of Bang (2) on blood lactic acid in exercise, and of Bugnard (6) and v. Euler (20), working in Hill's laboratory on various aspects of the heat relations of muscle. Gemmill (29) has reported a linear relation between work done and oxygen consumed by isolated frog muscles; this relation is to be expected without reference to whether contraction is fundamentally anaerobic or oxidative, since only the over-all relations are concerned. Flock, Ingle and Bollman (28) conclude from their studies on isotonic contraction of rat muscles *in situ* that contraction is fundamentally oxidative and that the appearance of lactic acid is due to an initial phase of oxygen deficiency.

There is inherent in any chemical method of determining the rate of oxygen utilization by contracting muscle a time lag which is usually fairly long. The development of spectrophotometric methods of recording changes in the rate of oxygen uptake following the onset of contraction, depending on differences in the absorption spectra of the oxidized and reduced forms of oxygen carriers, has reduced this time lag. Urban and Peugnet (96) have studied the reduction of cytochrome *c* in frog muscle, Quensel and Kramer (77) have made observations on the rate

of reduction of oxyhemoglobin in its passage through contracting muscle in the anesthetized dog, and Millikan (71) has investigated the desaturation of muscle hemoglobin in contraction of the cat soleus *in situ* by a technique which reduces the time lag to 0.2 second. The significant finding in these investigations is that the onset of contraction is accompanied by a simultaneous increase in the utilization of oxygen. As Millikan has pointed out, this finding can be satisfied either by direct oxidative reactions, or by anaerobic reactions in contraction which are reversed rapidly by oxidative recovery processes. It is necessary, however, that any postulated anaerobic reaction be reversed with sufficient velocity to account for the steady state.

*Oxidative recovery rates from anaerobic reactions.* The three anaerobic reactions which need to be considered as possible direct sources of the energy for contraction in the presence of oxygen are: the formation of lactic acid from glycogen, the hydrolysis of phosphocreatine, and the hydrolysis of adenosine triphosphate. It becomes necessary to determine whether the oxidative reversals of these reactions take place with the velocity necessary for the maintenance of the steady state.

With respect to lactic acid, it must be noted that in Meyerhof's experiments, a recovery period of about 24 hours was necessary after 20 to 30 minutes of anaerobic activity (62). This indicates that the oxidative resynthesis of glycogen from lactic acid is very slow. Measurements of the rate of removal of lactic acid by frog muscle show that about 20 mgm. per cent per hour can be disposed of if the concentration present is sufficiently high (12). The last condition is necessary because the oxygen consumption and carbohydrate synthesis of resting muscle are raised by the addition of lactate to the suspending medium (93). Lactic acid formation per twitch by the frog sartorius contracting anaerobically amounts to 0.7 mgm. per cent (24). Hill and Kupalov (41) maintained a steady state of 23 twitches per minute with this muscle in oxygen atmosphere. The assumptions of the Meyerhof cycle would require the rate of lactic acid removal under these conditions to be 16 mgm. per *minute* instead of the 20 mgm. per cent per *hour* actually found. In rat muscles, lactic acid formation in the first minute of activity may amount to 250 mgm. per cent, whereas the maximum rate of glycogen formation in recovery was found to be 3 mgm. per cent per minute (27). To explain the steady state which these muscles maintained after the first minute of activity by the Meyerhof cycle concept would require the assumption that the resynthesis of glycogen is some 70 times as rapid during activity as during recovery. However,

it has been found in both rat (27) and rabbit (89) muscles, that the entire amount of lactic acid formed during a period of anaerobic activity is lost to the muscle during the subsequent recovery period without the appearance of a detectable amount of glycogen in its place. In the rabbit experiments, the total carbohydrate loss in the recovery period was appreciably greater than the amount of lactic acid formed in the contraction; in the rat experiments, no synthesis of glycogen could be detected until after the lactic acid content had been reduced to the resting level.

With respect to the oxidative resynthesis of phosphocreatine: the maximum rate found for this reaction in frog muscle is less than $\frac{1}{2}$ mgm. per cent of P per minute (68). To maintain the steady state of the experiments of Hill and Kupalov by anaerobic hydrolysis and oxidative resynthesis of phosphocreatine would require that the resynthesis take place at the rate of 8 mgm. per cent of P per minute, a rate 16 times greater than that actually observed. In iodoacetate-poisoned frog muscle under anaerobic conditions, phosphocreatine P is converted to hexosemonophosphate P at the rate of 0.3 mgm. per cent per twitch (55). The poisoned muscle supplied with lactate and oxygen can maintain a steady state of activity of 10 (60) or even 14 to 16 (31) twitches per minute, with very little disappearance of phosphocreatine (61). The maximum rate at which frog muscle, normal or poisoned, is able to reconvert hexosemonophosphate to phosphocreatine is 8 mgm. per cent of P per hour (12). To maintain the steady state of the experiments with added lactate by anaerobic formation of hexosemonophosphate and oxidative resynthesis to phosphocreatine would require that the resynthesis take place at the rate of 3 to 5 mgm. per cent of P per *minute* instead of the 8 mgm. per cent per *hour* actually found. In mammalian muscle, a steady state of 2 twitches per second is readily maintained (91). For the energy to be supplied by phosphocreatine hydrolysis and oxidative resynthesis would require the recovery process to take place at the rate of about 70 mgm. per cent of P per minute; the rate actually found following such a steady state was only 5 mgm. per cent per minute (81).

With regard to the resynthesis of adenosine triphosphate, the only data available are for mammalian muscle. It has been found (28, 81) that this process is much slower than the resynthesis of phosphocreatine, and that there is no relation between the two processes.

From all these observations it can be seen that the hypothesis that contraction is anaerobic in the presence of oxygen requires the *ad hoc*

assumption that, at the same oxygen tension, the oxidative reactions take place with much greater velocity in the interval between two successive twitches than they do after the last twitch of the series. If the degree of saturation of the enzyme system concerned were the factor controlling the rate of resynthesis, the opposite relation would be anticipated, since the determinations of removal rate were made with much higher concentrations of substrate present than would be the case under steady state conditions.

With regard to the question of what substances undergo oxidation to furnish the energy for contraction, it must be pointed out that conditions in the normal animal are not suited to the determination of possible mechanisms. It cannot as yet be said with any certainty whether lactic acid, glucose, or hexosemonophosphate is the substance which undergoes oxidation. In the iodoacetate-poisoned muscle, lactic acid is oxidized even when only the normal resting concentration is present (55). It is therefore not unreasonable to assume that normal muscle can also oxidize this substance. Glucose is readily oxidized by the resting muscle poisoned with iodoacetate (3), but there have been no reports on whether glucose oxidation will support the contraction of the poisoned muscle. Ketone bodies can apparently be utilized for contraction, in amounts proportional to the oxygen consumption (5). The evidence with respect to neutral fat or fatty acids is not conclusive.

*Mechanism of anaerobic contraction.* In anaerobic contraction, the observed changes consist of: *a*, formation of lactic acid from glycogen; *b*, decrease in phosphocreatine content; *c*, increases in inorganic phosphate and hexosemonophosphate contents.

There have been two interpretations of these observed changes. One, which has been derived from the study of the enzyme systems present in cell-free muscle extracts, presents the phosphate interchanges and the formation of lactic acid as interrelated aspects of the mechanism for the energy supply in anaerobic contraction. This formulation is an integration of the work of the Cori, Embden, Meyerhof, Needham, Parnas and Warburg schools. The essential reactions are considered to be the following: glycogen reacts with inorganic phosphate to form glucose-1-phosphate, which rearranges to glucose-6-phosphate. This reacts with adenosine triphosphate to form hexosediphosphate and adenylic acid. The adenosine triphosphate is resynthesized by reaction of the adenylic acid with phosphocreatine, while the hexosediphosphate is split into two molecules of triosephosphate. Both glyceric aldehyde phosphate and dihydroxyacetone phosphate are formed. From this

stage on, there are two pathways. The one, which has been found in both muscle and yeast extracts, involves the oxido-reduction of triosephosphate to phosphoglyceric and glycerophosphoric acids; the former then rearranges to phosphopyruvic acid which transfers its phosphate group to the adenylic acid which has arisen from the formation of additional triosephosphate. The resulting pyruvic acid reacts with the triosephosphate to form lactic acid and phosphoglyceric acids, the latter then entering the reaction cycle again. The creatine which appeared at an earlier stage is rephosphorylated, either directly by adenosine triphosphate or by phosphopyruvic acid.

The alternative pathway, which to date has been described only for yeast extract—attempts to find it in living yeast were not successful—involves the *non-enzymatic* addition of phosphate to the aldehyde group of glyceric aldehyde phosphate, the oxidation of this to the 1:3 diphosphoglyceric acid by diphosphopyridine nucleotide (cozymase), loss of the phosphate group on position 1, and rearrangement to phosphopyruvic acid. A schematic representation of these reaction cycles and the other reactions which have been found in cell-free extracts, is given by Burk (8).

The other interpretation of the anaerobic phase (81, 85) considers that the observed phosphate changes, the hydrolysis of phosphocreatine and the formation of hexosemonophosphate from phosphocreatine and glycogen, are independent of the formation of lactic acid. The primary reaction which yields energy for contraction under anaerobic conditions is the formation of lactic acid from glycogen by a pathway which does not involve transfer of phosphate groups from either phosphocreatine or adenosine triphosphate. Whenever this mechanism is inadequate, the formation of hexosemonophosphate from glycogen and phosphocreatine is used as a substitute or supplementary reaction. The function of phosphocreatine hydrolysis is that of supplying alkali to neutralize the lactic acid formed.

Analysis of the extract formulation shows that it does not account satisfactorily for the observed changes in phosphate distribution which accompany the formation of lactic acid in muscle. If completed and balanced equations for the reactions are written, the net change is equivalent to the conversion of one hexose unit and one molecule of inorganic phosphate to lactic acid and triosephosphate, with no net change in phosphocreatine, adenosine triphosphate, or hexosemonophosphate. But it is only in extracts in which lactic acid formation does *not* take place, i.e., when an enzyme poison such as iodoacetate or

fluoride is present, that there is a decrease in inorganic phosphate and a formation of triosephosphate. In extracts in which glycolysis does take place, there is always an increase of inorganic phosphate and a loss of phosphocreatine, and in anaerobic contraction there is usually an increase in the hexosemonophosphate content at the expense of phosphocreatine P. If hexosemonophosphate is only an intermediate in the formation of lactic acid, it is difficult to see why it should accumulate most rapidly when the rate of formation of lactic acid is greatest, yet this is the situation found in anaerobic contraction, both in frog (24) and mammalian (91) muscle.

The data obtained on the iodoacetate-poisoned muscle contracting anaerobically are also at variance with the requirements of the extract formulation and the known effects of iodoacetate in extracts. The reactions which this poison have been found to inhibit in extracts are the dehydrogenation of triosephosphate to phosphoglyceric acid either directly (30) or through intermediate formation of diphosphoglyceric aldehyde (1), and the direct resynthesis of adenosine triphosphate from adenylic acid and phosphocreatine (45). The conversion of hexosediphosphate to triosephosphate is not affected, with respect to either the rate of reaction or the equilibrium point reached (66). On this basis, the poisoned muscle contracting anaerobically should show formation of hexosemonophosphate from glycogen and inorganic phosphate, followed by conversion of the monophosphate to the diphosphate by reaction with adenosine triphosphate, and then conversion of part of the hexosediphosphate to triosephosphate. The actual order of events, however, is: reaction of glycogen with phosphocreatine to form hexosemonophosphate, and conversion of the monophosphate to the diphosphate by reaction with either inorganic phosphate or adenosine triphosphate. The third reaction, conversion of part of the hexosediphosphate to triosephosphate, does not take place as long as the cell membrane is intact (83).

These data on normal and iodoacetate-poisoned muscle show that the formation of hexosemonophosphate is supplementary to, or substitute for, the formation of lactic acid, rather than an intermediate stage in its formation. Additional evidence that the two reactions are independent of each other is furnished by the experiments of Kerly and Ronzoni (46) on the effects of varying the pH of the suspending fluid on the resting anaerobiosis of frog muscle. In a medium of pH 6.0, hexosemonophosphate was formed, but no lactic acid, as long as any phosphocreatine remained. When this was exhausted, lactic acid for-

mation took place, but the *accumulated hexosemonophosphate did not disappear.* In a medium of pH 9.0, lactic acid was formed, but no hexosemonophosphate. It was also found (79) that when muscles which had been kept at pH 6.0 until hexosemonophosphate had accumulated and all remaining phosphocreatine hydrolyzed, were transferred to a medium of pH 9.0, the ensuing formation of lactic acid did not lead to loss of hexosemonophosphate.

Additional evidence in point is furnished by the effects of caffeine (13), epinephrine (35), and dinitrophenol (78) on resting anaerobiosis of frog muscle. All of these lead to increased lactic acid formation, and the epinephrine also causes hexosemonophosphate formation. Caffeine and epinephrine together give an additive effect, but with dinitrophenol and epinephrine there results the same lactic acid production that would be caused by dinitrophenol alone, together with markedly augmented formation of hexosemonophosphate (14).

It should be pointed out that the formation of hexosemonophosphate is a means for conserving the glycogen supply of the muscle, for this substance can readily be re-converted to glycogen within the muscle, either under steady state conditions (91) or in recovery (89). Lactic acid, however, is lost from the muscle by diffusion into the blood stream (28, 89). This glycogen conserving mechanism is most in evidence in both frog (24) and mammalian (91) muscle when the intensity of anaerobic work is the greatest.

The observation that a certain mechanism is present in muscle extracts by which lactic acid can be formed, and the finding that relations in the intact muscle are at variance with this mechanism, is not without analogy. One need only recall that tissue slices oxidize the natural *l*-enantiomorphs of the amino acids, while tissue brei oxidizes only the unnatural *d*-forms. The destruction of the cell membrane has resulted in the loss of a property manifested by the intact cell and the appearance of one not shown by the intact cell.

There is no doubt that muscle extracts *can* form lactic acid by mechanisms involving P transport by phosphocreatine and adenosine triphosphate, but this falls short of being proof that the intact muscle *does* form lactic acid by this mechanism. The development of radioactive isotopes has made it possible to trace these interchanges with certainty. Meyerhof (63, 69) and Parnas (75) have shown that the enzymes in muscle extract do not differentiate between radioactive phosphorus and the ordinary stable form. Application of the same technique to the intact muscle of the cat *in situ* (84) has shown definitely that neither

phosphocreatine nor adenosine triphosphate acts as P transporter in the formation of lactic acid in contraction. The only changes of phosphorus compounds found were those which are generally seen: hydrolysis of some phosphocreatine, and conversion of some phosphocreatine P to hexosemonophosphate. These findings also demonstrate that the hexosemonophosphate which is normally present in muscle is not an intermediate in the formation of lactic acid by the intact cell.

The results of the *in vivo* studies with radioactive phosphorus do not give an unequivocal answer to the question of whether all phosphorylating mechanisms are excluded. They do not exclude the possibility of phosphorylation reactions in which phosphate transport takes place through other substances than adenosine triphosphate and phosphocreatine. Ohlmeyer (74) has presented evidence that cozymase can act as the sole P transporter in systems containing hexosediphosphate. Such transport would need to take place by way of the reversible conversion of cozymase, diphosphopyridine nucleotide, to coenzyme II, triphosphopyridine nucleotide, described recently by Adler *et al.* (1). This follows because of Meyerhof (63, 69) has shown by means of radioactive phosphorus that cozymase does not undergo any dephosphorylation in glycolyzing extracts.

There are two major difficulties in this mechanism. One is that participation of adenosine triphosphate is still involved, for it takes part in the formation of hexosediphosphate. The other is that no triosephosphate appears in iodoacetate-poisoned muscles containing large amounts of hexosediphosphate so long as the cell membrane is intact (83), although Meyerhof and Lohmann (66) state that the enzyme poison is without effect on this conversion in extracts. This finding indicates that the mechanism of iodoacetate inhibition of glycolysis is different in the intact cell from that in extracts, and also that different reactions are involved in the formation of lactic acid in the two systems.

*The Pasteur reaction and the Meyerhof cycle.* The concept formulated by Meyerhof (62) of the mechanism by which oxidative processes prevent the accumulation of anaerobic metabolites is based on the experimental finding that the *extra* oxygen consumption of a recovering muscle is sufficient to oxidize only a part of the lactate which disappears. It is postulated that lactic acid formation takes place to the same extent in respiration as in anaerobiosis, and that respiration supplies the energy for the resynthesis to glycogen of the major part of the lactic acid formed. If the R.Q. of the muscle is 1.0, it would seem more reasonable to relate the lactic acid disappearance to the entire oxygen consumption rather

than to that fraction in excess of the oxygen consumption of a resting muscle. Burk (7) has shown that on this basis the oxidative quotient (lactic acid disappearing divided by lactic acid oxidized) is reduced to 2 instead of Meyerhof's value of 5. From the heat relations involved (7) it can be shown that the value of 10 would be required to account for the steady state, on the basis of 100 per cent efficiency for the resynthesis reaction. An efficiency less than this would mean that lactic acid or equivalent anaerobic metabolites, such as the hydrolysis products of phosphocreatine or adenosine triphosphate, must accumulate in continued activity, or even at rest. The oxidative quotient of 5 would thus mean 50 per cent efficiency for the resynthesis reaction, and consequently that half as much lactic acid should appear in the presence of oxygen as in its absence. This condition manifestly does not hold.

On the other hand, Shorr (93) has obtained high oxidative quotients, in some cases far above the theoretical maximum, with R.Q.'s approaching the level of fat oxidation. Such values are without significance in terms of the Meyerhof cycle concept, since the connection between carbohydrate oxidation and carbohydrate synthesis is absent. It has also been shown (3) that skeletal, cardiac, and smooth muscle poisoned with the minimal effective concentration of iodoacetic acid, maintain an R.Q. of 1.0 in glucose medium, and that the glucose disappearance is equivalent to the oxygen consumption. These theoretical and experimental considerations make it unnecessary to postulate either the anaerobic phase of the Meyerhof cycle or the common intermediate in the anaerobic and oxidative pathways of carbohydrate catabolism. The evidence at hand shows that the synthesis phase of the cycle is potentially present, but it does not have the significance assigned to it by Meyerhof, since it takes place only in isolated tissue systems, but not in muscle with normal circulation.

The Meyerhof cycle concept has led to speculation on the mechanism by which glycolysis, or any fermentation, for that matter, is inhibited by respiration. It is evident that some factor in cell organization imposes limitations on both the respiratory and glycolytic activity of cells. Muscle brei shows much greater lactic acid formation than intact muscle does in the absence of nitrogen, and also shows a much higher oxygen consumption than intact resting muscle. Lipmann (51) has presented evidence for a reversible oxidative inactivation of the glycolytic enzyme system. The validity of this attractive hypothesis is, of course, independent of the Meyerhof cycle concept. Either as it stands,

or with suitable extensions, it may be capable of explaining why certain enzyme poisons (CO, ethyl isocyanide, various dyes) can raise the level of aerobic glycolysis to the anaerobic level without decreasing respiration. The preliminary note by Stern *et al.* (95) on the existence of a "Pasteur agent" may offer the possibility of being this extension of the Lipmann theory. This must await further clarification and the publication of the experimental details. To be valid, it should be capable of explaining why muscle, at rest (48) or during the steady state of activity (91), shows a glycolysis the magnitude of which is related to the oxygen consumption. This continuous glycolysis amounts to about 10 per cent of that which would be found at the same level of activity under anaerobic conditions.

Careful analysis of Pasteur's (76) statement on the relation between respiration and fermentation shows that it does not contain, either explicitly or implicitly, the idea that continuous anaerobic breakdown of carbohydrate is taking place in the presence of oxygen. Pasteur took the position that the living cell utilizes oxygen insofar as it is available, and that fermentation reactions take place only when the supply of oxygen is inadequate to support a fully aerobic metabolism. Yeast, the subject of Pasteur's investigations, clings very tenaciously to the respiratory pathway of metabolism. For example, Warburg and Kubowitz (97) found that at 10°C., the oxygen tension must be lowered to 0.2 mm. Hg to lower the respiration of yeast by 50 per cent. If the view be adopted that respiration is the fundamental metabolic activity, and that anaerobic reactions are resorted to when, and only when, the supply of oxygen is inadequate to meet the metabolic requirements of the cell, much of the mystery surrounding the term "Pasteur reaction" vanishes. An emergency anaerobic mechanism for muscular contraction is obviously necessary, for any appreciable muscular effort involves an initial period of deficient oxygen supply (47). Until the circulation becomes adequate, anaerobic reactions must be called upon to a certain extent, but this does not mean that they are used to the exclusion of direct oxidative ones when sufficient oxygen is available.

*Thermodynamics of muscular contraction.*[1] It must be pointed out that Hill's (36) finding that the relation between heat liberated and tension developed in contraction is independent of the presence of oxygen, is one which is to be anticipated on the basis of the second law of thermodynamics. In the contracting muscle, as in the steel spring and

[1] The writer is indebted to Dr. H. L. Johnston, professor of physical chemistry, Ohio State University, for this analysis of the thermodynamics of muscle.

the rubber band, the relation between tension developed and heat liberated depends only on the force law involved, i.e., the mathematical statement which expresses the relation between the change in length and the force necessary to produce it. It is immaterial from the thermodynamic viewpoint whether the muscle is actively contracting or being passively stretched, and it is also immaterial whether the source of the energy for contraction is some physical process or chemical reactions. Different sets of chemical reactions which may be the source of the energy for contraction under different conditions can modify the ratio of heat to tension only if they produce changes in the form of the force law or in the numerical values of the constants therein.

It can therefore be seen that if different relations between heat and tension were to be found under aerobic conditions than under anaerobic, this would be a demonstration that different sets of chemical reactions were taking place under the two conditions. On the other hand, if the same heat-tension relations are found, it serves to demonstrate merely that the different chemical processes do not give rise to any change in the force law of the muscle. Only the heat liberated during the mechanical event enters into the matter; the heat liberated after contraction and relaxation are over is a function of the living cell and not of the thermodynamic system. The experimental data show that in every case which has been investigated, the ratio between heat and tension is unchanged. These cases include: normal muscle in oxygen and nitrogen (36), iodoacetate-poisoned muscle in oxygen and nitrogen (21), and curarized muscle in nitrogen (21). The chemical reactions taking place in these muscles are all different except in the cases of the normal and iodoacetate-poisoned muscles in oxygen. It can therefore be stated that the constancy of the relation between heat and tension cannot be used to prove that there is a single reaction, or set of reactions, supplying the energy for contraction under all possible conditions. The data obtained are useful for determining the force law of muscle, but beyond this one cannot logically proceed.

*Post-contraction phenomena.* There remain to be explained the heat liberation shown by muscle in the period after the completion of contraction and relaxation, and the other phenomena of the post-contraction period. Hill interpreted this post-contraction heat as demonstration of the oxidative recovery from anaerobic contraction. It must be pointed out, however, that smooth and cardiac muscle do not show this phenomenon, and that striated muscle shows it in the absence of oxygen. Analysis of the steady state of activity of striated muscle shows dis-

crepancies between the requirements of Hill's theory and the observed phenomena. If the steady state were to be maintained by anaerobic contraction and oxidative recovery, then the amount of recovery accomplished between two consecutive contractions must be equivalent to that after a single isolated contraction. Otherwise there would be an accumulation of anaerobic metabolites and the steady state would not be present. Now the duration of the liberation of the recovery heat after a single twitch of frog muscle at room temperature is about 40 seconds (38); unless repeated stimulation results in an acceleration of the recovery process, then an activity level greater than one twitch per 40 seconds should lead to the accumulation of anaerobic metabolites. Cut Hill's (38) measurements show that the total time required for the evolution of the recovery heat after $n$ twitches is practically $n$ times as great as after a single twitch, and that the total heat liberation is also $n$ times as great as after a single twitch. This shows conclusively that repeated stimulation does not lead to any significant acceleration of the "recovery" process. Yet Hill and Kupalov (41) were able to maintain a steady state of 23 twitches per minute in the frog sartorius in oxygen, instead of the $1\frac{1}{2}$ per minute theoretically possible. At 0°C., the time for complete evolution of the recovery heat after a single twitch is 30 to 40 minutes (40) and the oxygen consumption shows the same time course (43). On this basis, the maximum steady state activity which should be possible at this temperature would be only two twitches per *hour* instead of the two per *minute* that Hill found to be feasible.

Another observation used by Hill as evidence of oxidative recovery from anaerobic contraction is the extra heat liberated when oxygen is admitted to a muscle which has previously given a series of twitches in nitrogen (38). But this increase in metabolism can be brought about by many processes which do not involve any antecedent contraction. It is observed when lactate is added to the solution bathing the muscle (93), and it appears when the high lactic acid content is produced by resting anaerobiosis. It is seen also after exposure to an atmosphere of carbon dioxide (79). Under these conditions there is hydrolysis of phosphocreatine and formation of hexosemonophosphate. The excess metabolic activity on admission of oxygen to such a muscle is evident as long as phosphocreatine is being resynthesized from its hydrolysis products (79). This effect of the hydrolysis products of phosphocreatine in producing an augmented oxygen consumption can also be obtained with muscle brei (4).

The magnitude of the post-stimulation heat is subject to wide fluctua-

tions under different conditions. It is greater in frog muscle after tetanus than after twitches (9); in mammalian muscle it is much greater at room temperature (23°) than at 37° (11), and it is much greater in mammalian muscle than in frog muscle at the same temperature (11). When the resting metabolism has been elevated by caffeine, the magnitude of the post-stimulation excess of metabolism is much reduced (92). These observations make it difficult to accept the proposition that the post-stimulation heat represents the oxidative recovery from an anaerobic contraction process, for they necessitate the assumptions that the efficiency of recovery is subject to very wide fluctuations with temperature and type of activity, and that it is quite different in frog muscle than in mammalian. It is well known (22) that nerve shows a similar post-stimulation excess of metabolism, amounting to perhaps 20 times the metabolism during the stimulation period. If this did represent a recovery process in nerve, then the efficiency would reach the very low figure of 5 per cent.

Post-stimulation heat and chemical changes, moreover, are not limited to contraction in the presence of oxygen. They occur, to lesser extent, under anaerobic conditions (33). There would seem to be an essential similarity between the post-stimulation excess of oxygen consumption, the post-stimulation formation of lactic acid by frog muscle contracting in nitrogen (18), and the post-stimulation breakdown of phosphocreatine shown by the iodoacetate-poisoned frog muscle contracting in nitrogen (57). In all these cases, there is a continuation into the post-contraction period of the metabolic reactions that took place during the contraction.

There has been offered an alternative interpretation of these phenomena of the post-contraction period, namely, that the tissue which has been stimulated does not return instantaneously to the resting metabolic level (88). The magnitude of this post-stimulation excess of metabolism is related to the duration and intensity of the activity. This explanation is equally valid in interpreting the enormous post-stimulation heat production shown by nerve. The effect is merely of a different order of magnitude than that in muscle.

On this basis, the "oxygen debt" in exercise is merely the manifestation in the entire organism of a phenomenon which is shown by the isolated muscle or nerve. Hill (42) applied the term oxygen debt to the excess oxygen consumption above the resting metabolic level which is found after exercise, and which is related in magnitude and duration to the rate and duration of the exercise. This excess oxygen consump-

tion can be separated into a phase immediately following the cessation of work, which shows rapid decrement, and one which shows slow decrement and long duration. Both phases are present independently of whether there is a rise in the level of lactic acid in the blood. Hill *et al.* (42) considered that the rapid phase represents the resynthesis of lactic acid to glycogen within the muscles in which it was formed, and the slow phase represented the same process taking place in the other tissues into which the lactic acid had diffused. It is known, however, that the limited resynthesis of lactic acid to glycogen which does take place in muscles is a very slow process. Margaria *et al.* (58) assign to the slow phase the function of lactic acid removal, and regard the rapid phase as the oxidative resynthesis to its precursor of some anaerobic metabolite other than lactic acid, since the rapid phase is present even when no increase in blood lactic acid is found. However, no anaerobic reaction has been found in muscle which has the properties of rapid oxidative reversal called for by this hypothesis (81). Also, it has been shown (90) that the lactic acid content of the blood after a short period of steady state activity, such as that used by Margaria *et al.*, is very much lower than that of the muscles themselves. The other phase of the "oxygen debt payment" far outlasts in time that which is required to reduce the lactic acid content of the blood and muscles to the resting level. In view of all these difficulties encountered in relating the post-exercise increase in oxygen consumption to the oxidative removal of anaerobic metabolites, it may be more reasonable to regard it as essentially the same phenomenon that an isolated muscle shows after contraction in oxygen, as a gradual return to the resting metabolic level.

Although there have been many advances in our understanding of the chemistry of muscular contraction during the past decade, many problems still remain to be solved. The mechanism of lactic acid formation within the muscle fiber still remains to be discovered, and the function of adenosine triphosphate awaits elucidation. The whole field of the oxygen carriers involved, and the intermediate stages in the oxidation of the various substrates is just now beginning to be explored. The cytochrome systems are undoubtedly of greatest importance; in fact, Stannard (94) finds that azide and hydroxylamine, specific poisons for this system, do not affect the resting oxygen consumption, but abolish the increase due to stimulation or to caffeine. The participation of the flavine systems is indicated by the spectrophotometric observations of Urban and Peugnet (96).

Finally, and most elusive of all, there remains the question of the

mechanism by which the muscle transforms the chemical energy derived from oxidative or anaerobic reactions, into the mechanical work which is its function in the body.

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# RECENT DEVELOPMENTS IN HISTOCHEMISTRY*

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Histochemistry deals with the chemistry of tissues. It differs from conventional biochemistry by referring chemical substances to cells and to the pericellular spaces, which together comprise a tissue. Thus, in the analysis of skeletal muscle for inorganic ions, a histochemical problem is the determination of the quantities of each ion present at a particular moment and even in particular places in the muscle fiber, in the tissue fluid space around it and in the connective tissue cells. Again, in studies on nucleic acids, a histochemical problem is the determination in a variety of cells in different functional (and genetic) states of how much is present in the chromosomes, nucleoli and nucleoplasm of the nucleus and in the cytoplasm. The emphasis throughout is on the morphological localization of chemical substances in recognizable tissue space, in cells, and indeed, in the structures of nucleus and cytoplasm. Thus, histochemistry includes certain aspects of cytochemistry. The aims of histochemistry are 1, to define quantitatively the relations of chemical substances located intracellularly with those in their immediate environment, and 2, to determine quantitatively the relations of intracellular chemical entities to the morphological organization of the cell. It is obvious that to the extent to which these goals are approached, new light will be thrown on the functional association between cells in the same or different tissues and on the rôle played by cellular components in metabolic mechanisms.

It is needless to note that the results achieved by histochemistry up to the present time fall far short of the end in view. There are many reasons for this lack of agreement beteeen aspirations and achievements. 1. The concentration of the constituent sought in tissues is usually close to the limit of detectability by the methods employed, when it does not fall below it. 2. Numerous substances are closely bound to other confusing or interfering substances. 3. In both of the preceding classes, phenomena such as diffusion, solubility, denaturation, and inactivation loom large as disturbing factors. 4. In the attempt to visualize selectively one substance present in an intimate and complex

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mixture, there are no guiding criteria against which to test the accuracy of, or determine the limitations of the methods employed. A very useful means of estimating the value of a histochemical method is quite pragmatic: those methods are useful which yield results that are in essence in agreement with, or confirmed by, directly related data obtained by means of simpler, chemically valid or physiological (functional) techniques. 5. Technical difficulties incline histochemical work toward a descriptive, qualitative state. It is noteworthy that in the last decade, some of the most significant advances in histochemistry are quantitative ones (see enzymes, nucleic acid).

In the broadest sense, good histochemical work need not be done only with the microscope. For example, the distribution of inorganic substances in striated muscle cells and in the intercellular tissue fluid is significant tissue chemistry, even though it was resolved for the most part by conventional biochemical analyses of whole muscles. Chemical analysis of tissue fluid aspirated from a skeletal muscle may be considered also as histochemistry. Also useful in determining the nature of the environment of the cells of a tissue are chemical analyses of lymph close to its source, of blood as it enters and leaves a capillary bed, etc. Chemical analyses of the fluid carefully withdrawn at known intervals from the lumen of the renal nephron is also histochemistry comparable with conventional histochemical approaches to the problem. Such chemical studies of the immediate environment of the cells of a tissue are reviewed in familiar physiological literature.

This article deals primarily with the more conventional aspects of the histochemistry of higher animals. The material presented is collected under various headings with numerous cross-references: 1, various aspects of metabolism; 2, organs or tissues, and 3, methods. The literature cited is not exhaustive—it was carefully selected from among the most significant contributions in each subject treated. With few exceptions, the period covered is the last decade. References to earlier literature are very adequately treated in Lison's *Histochimie Animale* and in the smaller sectional reviews mentioned in this paper.

INORGANIC IONS. *Chloride, phosphate + carbonate, potassium, calcium, magnesium.* There are two essential requisites for the detection of diffusible substances in tissues: 1. Tissues should be fixed in such a way as to prevent diffusion and preserve these substances very nearly in the position they occupied during life. 2. The particular substance investigated should be visualized in microscopic sections with no significant displacement. Chemical reactions, if used in this process,

should be specific and sensitive, and should avoid destruction or disruption of cellular or intercellular materials. Calcium, magnesium, and silicon are the only inorganic substances which have been detected by other than chemical means (see p. 245). The first condition has been satisfied by the reintroduction of Altmann's (1890) freezing-drying method (1, 33). Parts of animals are removed rapidly from the body and frozen in liquid air or in fluids cooled to temperatures below −125° C. (63). They are then dried in a high degree vacuum chamber kept at −14°C. to −62°C., depending on the substance being investigated (56, 63, 117). After drying, the blocks of tissue are embedded in paraffin and cut with a microtome. Sections of skeletal muscle and several other organs were prepared in this way and then tested for chloride, phosphate + carbonate, and potassium (39). In skeletal muscle, chloride was found only in the intercellular spaces and phosphate + carbonate was visible in the muscle fibers and between them. Potassium was distributed in the same way, but was present in vastly greater amounts in the muscle cells. These observations thus confirmed in a direct, though qualitative manner the distribution of these ions in skeletal muscle that was arrived at from biochemical data (24, 25, 62). These observations are important because they define the nature of the pericellular milieu in muscle and the permeability of the membrane of the muscle fiber. Studies on the distribution of chloride and phosphate + carbonate in the fundic mucosa are discussed on p. 257. A further study showed that when the formation of colloidal calcium phosphate is induced in the circulating blood, this substance is phagocytosed by macrophages of the liver, spleen and bone marrow, and later released (38). The uptake and release of the ingested particles were correlated with chemical studies on the concentration and state of calcium in the blood and in general with certain aspects of calcium metabolism (40, 96). The methods employed in these investigations have been criticized by Scott and Packer (121) as permitting a significant shift in the ions. Their criticism is based largely on theoretical grounds which were clearly defined by Gersh and Stieglitz (43) and by Lison (90). The reviewer, nevertheless, still regards his previous conclusions as valid, in view of his constant awareness of this possible source of error, and because of the uniformity in the results, the support of the essential conclusions by related data obtained with entirely different biochemical methods, and the absence of any demonstration to the contrary.

McLean and Bloom (95), using the v. Kossa method to demonstrate

bone salts in undecalcified serial sections of rat bones, report that in developing bone a zone of uncalcified matrix (osteoid tissue) is a minor phenomenon that is not constantly present and that the wide osteoid borders, the so-called physiological osteoid described in the literature, probably represent a diet deficient in calcium or phosphate. In the resorption of bone they were unable to demonstrate a removal of bone salts before solution of the matrix. In experiments with activated sterols and very large doses of parathyroid hormone, they found calcium salts, mobilized from bone, distributed through the marrow and up to the endothelium. They have been unable to visualize calcium salts passing from vessels to developing bone or cartilage. They have found no evidence of phagocytosis of bone salts by osteoclasts.

*Heavy metals.* There have been few significant histochemical studies of the rare or heavy elements occurring normally or introduced into the body for medical purposes. The chief reason for this is the insensitivity of the methods employed to demonstrate their presence, especially in view of the low concentration of the elements sought. New methods, or modifications of old ones, have been described in the last five years for the histological detection of bismuth (16, 18), gold (21, 101), silver (104), palladium (102), arsenic (17), iron (131), copper (103, 105), and lead (125).

*Microincineration.* Microincineration of thin sections of tissues has been developed by Policard and extended to the study of developing, neoplastic, and normal and pathological adult material (117, 118, 119). It has been found that with appropriate care the morphology of the total ash residue for each organ, tissue, and cell is as characteristic as the appearance of the structures in stained preparations. No significant alterations in distribution have been described in different functional states (119). Such changes are, however, recognizable in embryonic and pathological material. In most cells, the ash deposit closely resembles stainable structures; finer details (as mitochondria, neurofibrillae, etc.) escape representation as such in the final picture. Apart from the identification of silicon by virtue of the double refraction of some of its compounds, no other elements could be localized with certainty in ashed preparations until the recent reports by Scott and Packer (120, 121). These workers were able to identify specifically Ca + Mg in "ashed" preparations of striated muscle by observing on the fluorescent screen of an electron microscope the sites which activate the emission of electrons on being heated to 700° to 800°C. These elements were found to be entirely or almost entirely localized in the muscle cells,

chiefly in the "contraction nodes", while little or none could be detected in the tissue fluid spaces. Movement of the more diffusible tissue elements in the stages preceding incineration was reduced to a minimum by preserving the tissue by freezing and drying. Evaluation of observations of incinerated preparations should be tempered by the knowledge that retraction, rolling and rupture of the section as a whole (or of parts of it) may occur during heating (133). Moreover, Godlewski (54) found by direct observation of sections during incineration that following the violent bubbling of paraffin that occurs at lower temperatures (up to about 150°C.), strong intracellular displacements take place as the temperature rises, with the formation of fissures, holes, masses, flakes, filaments and granules. Thus, according to these observations, the ash picture in a well-incinerated preparation may represent the final result of a whole series of previous rearrangements in the organic (and inorganic) constituents of cells that have taken place during the process of ashing the section. This intrinsic source of error may explain the failure after microincineration to detect physiological shifts in inorganic substances; it may also account for the paucity of ash visible in tissue fluid spaces and its absence from the glomerular space, etc. More direct observations of the events that take place during incineration are necessary for an evaluation of the possible artifacts that may take place in such preparations.

*Historadiography.* It has been found that certain cells and parts of cells exhibit differences in permeability to soft x-rays which can be recorded on photographic plates. For example, nucleoli absorb such x-rays more strongly than the rest of the nucleus, mucigen granules more than myelin or lipid inclusions, cells of the stratum germinativum of the skin more than striated muscle cells, etc. X-ray absorption is thought to depend on the presence in the region photographed of concentrations of elements with low atomic weight or on the presence of heavy metals. The use of this method confirmed the disposition, previously described by means of color reactions, for rather large deposits of bismuth, arsenic, and gold. The method lacks the specificity and precision so essential for critical histochemical work (19, 84, 85, 86, 132).

Of far more significance is the report of Dershem (22) who irradiated microscopic sections of undecalcified bone with nearly monochromatic x-rays arising as a fluorescence from a thin film of scandium activated by soft x-rays. Calcium will absorb emitted light of this particular wave-length almost specifically. The disposition of calcium is then determined from the clear areas of exposed photographic plates. The chief handicap is that the image can be magnified only slightly.

*Histospectroscopy.* Few significant histochemical contributions have been made by the adaptation of spectrographic methods for the analysis of biological materials (110, 122). The tissue elements capable of analysis have been so large as to make accurate histological localization impossible, except in the case of certain pathological specimens (98, 108, 109). Another limiting factor is that the identification of elements is not quantitative.

ENZYMES. *Pepsin, peptidase, urease, esterase, lipase, amylase.* Ingenious micro-methods were developed by Linderstrøm-Lang and his colleagues to determine in what cells certain enzymes occur, or, in the case of eggs or large unicellular organisms, in what part of the cell they are present. The technique has made possible also an attempt to localize certain other substances in tissues, such as hydrogen and chloride ions (see p. 256), and vitamin C (see p. 251). In the extensive studies on the gastric mucosa of the pig (only urease was studied in the dog), the preliminary operations were always the same. The whole stomach was emptied of its contents and chilled. Small plugs 2.5 mm. in diameter were bored out, and these were cut parallel to the surface in a cold chamber at a constant thickness (25 $\mu$). Enzyme determinations on one or two sections at definitely spaced distances from the surface made it possible to estimate enzyme content throughout the whole thickness of the mucosa. The tissue surrounding the hole formed by the removal of the plug was embedded in paraffin and sectioned serially. Cell counts were then made to determine the numbers of each cell type present at all levels of the mucous membrane. Curves of cell type population at each level were compared with those showing the enzyme content throughout the depth of the glands. Comparisons of such curves showed that the greatest peptic activity was present where the chief cells were most numerous. This is the only unequivocal evidence that these cells are the source of pepsin. Dipeptidase was also found in these cells. Slight peptic activity was present in the pyloric glands, where, in addition, the strongest dipeptidase concentration occurred. In both fundus and pylorus, urease was associated exclusively with the surface cells or with the connective tissue adjacent to them, or with both elements. In the cardia, possible traces of peptic activity and rather large amounts of dipeptidase were associated with the gland cells. Esterase seemed to be present in all cell types of all regions of the gastric epithelium and also, apparently, in the connective tissue. The highest dipeptidase activity was observed in the duodenum, particularly at the levels occupied by the glands of Brunner (49, 87, 88).

Similar analyses of the adrenal gland showed that high lipolytic

metanephros of the fetal pig (26). Here again the non-secretory stages did not show the differential distribution of the cytochrome oxidase system or the differences of potential that appear in later secretory stages between epithelium and connective tissue. These workers present an experimental approach and concept that is capable of being extended to other organs of the body, and other developmental processes (see Rulon, 1935).

A further study of the substance responsible for the large reducing capacity characteristic of the ciliary stroma identified the presence of at least two such reversibly oxidizable substances, one of which appears to be ascorbic acid. Vitamin C was found by the silver method to be richer in the stroma than in the epithelium and to be uniformly distributed in the former. Depletion of the vitamin C content of the ciliary body altered the oxidation-reduction potential of the ciliary tissues, the ionic electric current between epithelium and stroma (as demonstrated by the movement of basic dyes) and perhaps the rate of transfer of water. These properties were more or less restored to normal very shortly after the administration of the vitamin (32).

Methods have been described for the localization in tissues of succinodehydrase (123) and phosphatase (55, 130). The presence of phosphatase was noted particularly in the mucous membrane of the intestine, the proximal convolution of the renal nephron, the seminiferous tubules, ovarian follicles, ossifying cartilage and the endothelium of certain regions. A combination of the use of appropriate material and micro-methods for amylase and protease such as those introduced by Pickford and Dorris (106) should extend greatly our knowledge of the factors involved in the development and secretion of these enzymes in gland cells (23).

SULFURIC ACID ESTERS. *Heparin.* In the course of a very extensive study of the significance of the metachromatic staining of certain specific tissue elements with dyes like toluidine blue, Lison (92) gave good reasons for believing that this reaction is specific for complex sulfuric acid esters of large molecular weight, such as chondroitin, mucoitin, etc. This made it possible for the first time to attach at least some chemical significance to the large number of studies on cartilage, and other connective tissue derivatives, and "mucous" gland cells, both of which had been known to stain purple with toluidine blue. Later, Holmgren (67) published additional data which suggest that it may be possible to differentiate sulfuric acid esters of low molecular weight from the larger polyesters. This makes feasible an investigation of cytological phenom-

ena associated with the first appearance of precursors of collagen and cartilage. A reinvestigation of the amorphous ground substance of the connective tissue, which stains metachromatically, should lead to a chemical interpretation of this material. Heparin, a complex sulfuric acid ester, was found also to be precipitated and stained metachromatically by toluidine blue. A series of investigations by Jorpes, Holmgren and Wilander (67, 68, 78, 79, 136) disclosed a close parallel in the heparin content of various organs, in the same and different animals, with the number of mast cells present. They believe the granules of these cells, which stain purple with toluidine blue, contain heparin. The tendency of mast cells to be associated with capillaries, and the loss of their granules in peptone shock (when the heparin content of the blood is increased), is further evidence in support of their argument.

VITAMINS. The fact that vitamin C reduces silver salts in acid medium enabled Giroud and his co-workers to localize this compound in tissues (45, 46). The reaction suffers from several grave defects: 1. It is rather insensitive, being negative when the concentration of ascorbic acid is less than one part in 50,000. 2. Certain inhibiting substances (glutathione, epinephrine, cysteine, etc.) reduce the sensitivity still further or inhibit the reaction, notably in the medulla of the adrenal gland. 3. Since the silver salts are far less permeable than vitamin C, the finer localization is open to question, particularly when the former are injected by way of the blood vessels. For this reason it is difficult to attach great significance to the reported distribution of the vitamin in relation to mitochondria, Golgi apparatus, or even in the ground substance of the cytoplasm (47). For the same reason the speculations of Joyet-Lavergne (80) on the respiratory function of the mitochondria are somewhat premature. 4. The reaction is not quantitative. The last objection is largely overcome by the technique introduced by Glick (48). This method, in addition, is more sensitive and specific, and freer from errors due to interfering substances. Using the principles of Linderstrøm-Lang, Glick and Biskind were able to refer their determinations, in a statistical sense only, to individual cells in each zone. In the hypophysis, the cells of the infundibular process were found to be richest in vitamin C; its concentration in the cells of the pars intermedia was greater than in those of the anterior lobe (52). In the adrenal gland, the cells of the outer fasciculata contained the most ascorbic acid, the amount decreasing in the cells of the inner fasciculata, reticularis and medulla; none was found in the capsule or glomerulosa (50). In the thymus, the concentration of vitamin C per unit of parenchyma re-

reaction is specific for desoxyribose nucleic acid (90), a weaker negative reaction cannot be interpreted as indicating the absence of this compound (9). Moreover, the positive color reaction is so intense as to make quantitative estimations impossible. Color reactions of nucleoproteins were studied from this point of view by Kelley who offered a mechanism for such staining reactions with basic dyes (82, 83). Extensive work on nucleic acid metabolism in cells awaits the development of some such type of staining reaction, which will make possible unequivocal quantitative estimations. Ultraviolet absorption methods are technically difficult, costly and time-consuming. When staining methods are standardized on appropriate test material with ultraviolet absorption studies, progress unquestionably will be more rapid and general. It should be noted that the staining mechanism proposed by Kelley should apply equally well to the localization of nucleic acid compounds in the cytoplasm.

*Iron, zinc.* Further efforts have been made to evaluate reports on the presence of iron in the nucleus. Policard (107) believes that iron in the nucleus, as reported in the early studies by Miescher *et al.*, is derived from the remnants of cytoplasm which were carried along with the separated nuclei. Miyake (97) and Yakusizi (137) have recently claimed that zinc is also present in their similarly crudely separated nuclear material.

*Enzymes.* Peptidase was found to be almost or entirely absent from the nucleus (see p. 248). The cytochrome oxidase system has been reported frequently as not being present in the nucleus, but the results are difficult to interpret (see p. 249).

CYTOPLASM. *Ground substance.* The ground substance of the cytoplasm was shown to contain peptidase and catalase evenly distributed throughout the cell (see p. 248). R. R. Bensley (4) has studied the properties of an extract of liver cells prepared with 10 per cent sodium chloride, which he calls plasmosin. Chemically this is a poorly defined substance or group of substances (probably present in the ground substance of the cytoplasm) which he believes may be the basis for such phenomena as reversible gelation, thread formation, fibrillae and other grades of viscosity in the cytoplasm or in some of its structures. The relation to other fiber-forming chemically pure substances such as myosin or nucleic acid is unknown.

*Mitochondria.* Following preliminary studies (5) on the behavior of mitochondria in liver cells toward various solvents after the organ had been frozen and dried, came R. R. Bensley's remarkable feat of

separating mitochondria from fresh and frozen-dried livers (3). To what extent the mitochondria are contaminated or infiltrated with other cytoplasmic substances is unknown. Mitochondria were isolated in sufficiently large numbers to be analyzed chemically. They were shown to contain proteins, glycerides, cholesterol and perhaps also lecithin. These substances were believed to be highly and evenly dispersed in each mitochondrium though perhaps more concentrated on the peripheral than in the central parts of each organoid.

ENDOCRINE GLANDS. *Adrenal cortex.* In spite of the great need for histochemical studies of the endocrine glands, few significant contributions have been made in recent years. Bennett (2) localized strongly reducing substances (including ketones) in the outer fascicular, lipoid-rich, "secretory" zone of the adrenal cortex by forming phenylhydrazones after removal of ascorbic acid from sections. He believes that the yellow compounds formed in this way represent the phenylhydrazones of a mixture of acetone and alcohol soluble ketones which include the biologically active corticosterones (corticosterone, desoxycorticosterone and dehydrocorticosterone). It should be recalled that the relationship of these chemical substances to the native cortical hormone(s) is not entirely clear. Accepting the conventional concept of growth and repair in the adrenal cortex, he believes that the cortex peripheral to the outer fascicular region in the cat is presecretory, while the deeper parts are postsecretory. No studies on glands in different functional states have yet been reported.

*Thyroid gland.* Gersh and Caspersson (42) identified organic iodine compounds (thyroxine + diiodotyrosine) and total protein in the colloid of individual follicles in the thyroid gland (see p. 252). This was achieved by determining that the shape of the ultraviolet absorption curves of colloid in microscopic sections is identical with the curves of proteins containing cyclic amino acids such as tyrosine and tryptophane (with their peak at 2800 Å), thyroxine and diiodotyrosine (with their peak at 3200 Å), and thyroglobulin (with peaks at 2800 Å and 3200 Å). A quantitative comparison of the height of the absorption curve (at 2800 Å and 3200 Å) of colloid in different follicles of the same gland showed that there was a great variability in the protein and organic iodine concentration though it was uniform throughout each follicle at any one moment. A change in the mean chemical constitution of the colloid in different physiological states was demonstrated. The results can best be explained by assuming that intrafollicular colloid is formed and reabsorbed continually in the normal and in certain other

functional states, and that in extreme activity organic iodine compounds are secreted basally without entering the lumen of the follicle. Certain cytoplasmic droplets were tentatively identified as consisting of organic iodine compounds.

*Pancreas.* In the islets of Langerhans, further attempts were made to correlate the solubility (fixability + stainability?) of cytoplasmic granules in the A and B cells with the presence of lipocaic and insulin (S. H. Bensley (6) and S. H. Bensley and C. A. Woerner (7)). It should be emphasized that solution by reagents is not an adequate criterion for identifying chemical substances as complicated as proteins (see Caspersson (10)) and that the concept of cytoplasmic granules as activity carriers does not rest on too firm a basis (see p. 249). The criteria employed in these studies have more validity when considered as morphological rather than as chemical in nature.

Certain chemical studies on the pancreas which are mentioned rarely in reviews on the subject should be emphasized. E. and G. Hammarsten and Olivecrona (57) reported that whereas the normal dog's pancreas contains appreciable amounts of pentose nucleic acid, only traces are found after duct ligation has resulted in complete or almost complete degeneration of the acinar tissue. This was confirmed later by Jorpes (77) when he found that islets in certain fish contain far less nucleic acid than the zymogenic tissue of the pancreas, though the former are richer in phospholipids. These supplement earlier studies showing that islet tissue contains all or almost all the insulin in the pancreas (75, 93, 94, 135).

The distribution of vitamin C in the endocrine glands has been summarized above (see p. 251).

Exocrine glands. *Stomach.* Recent histochemical work on the stomach has been concerned chiefly with the determination of the types of cells which produce or contain enzymes (see p. 247), and with the problem of the site and mode of formation of hydrochloric acid. Preliminary studies of the distribution of chloride and of "acid" by Linderstrøm-Lang, Holter, and Ohlsen (87) are necessarily inadequate since the methods they employed cannot prevent (rather, they encourage) the diffusion of both of these substances between the time the organ is removed and the frozen sections are analyzed. The determination of the hydrogen ion concentration of the water in the cytoplasm of the gland cells, canaliculi and contents of the lumina of the fundic glands has been limited because few indicators penetrate the epithelium in sufficiently high concentration to be useful (99). Neutral red has been

used most extensively (58, 64). According to Lison (89) it is difficult to interpret results observed with this indicator because one does not know how they are affected by certain sources of error such as that due to the presence of proteins and salts, to adsorption, to solution of the indicator in lipids, to the formation of intracellular inclusion vacuoles, and to what has been called the "metachromatic error". It is difficult, then, to accept the conclusion derived from work with neutral red (chiefly) that hydrochloric acid is formed in the outer portion of the lumen or in the foveolus of the gastric glands by hydrolysis of a protein chloride formed, stored, and secreted by the parietal cells, whose cytoplasmic water is claimed to be distinctly alkaline (58, 64). Equally inconclusive results were obtained by Lison (91) and Gersh (41). The latter author prevented post-mortem diffusion of chloride by freezing the gastric mucosa in liquid air and drying it in a vacuum at −60 to −62°C. The subsequent procedure visualized chloride in microscopic sections (see p. 244) in the lumen of the glands, in the tissue fluid spaces, and in the zymogenic granules of the chief cells but not in the parietal cells, even when hydrochloric acid was being actively formed. There is as yet no direct evidence that implicates the parietal cell in the formation of hydrochloric acid or that serves as a basis for deriving the mechanism by which it is formed.

*Liver.* In an analysis of the diurnal rhythm which occurs in the chemical composition of the liver of certain rodents and hedgehogs a Swedish group of investigators developed a method for visualizing the bile components (including bile pigments + bile salts) in the hepatic lobule (29, 30, 65, 66, 69, 70). Bile components are precipitated by $Ba^{++}$ included in the fixative, the precipitate being stained with acid fuchsin. In a comparison of the fluctuation in glycogen, fat and bile content of the liver these workers found that when the glycogen content of the liver is low and glycogen appears chiefly in the cells close to the central vein, the bile components are visible in the wide bile capillaries throughout the hepatic lobule with large amounts present in the parenchymatous cells as granules. When the glycogen content of the liver is high and glycogen is present in most cells of the hepatic lobule, the bile capillaries are narrow and chiefly those at the periphery contain a smaller amount of bile components; the latter cannot be seen in the gland cells or are visible there only in traces. Indirect evidence suggests that a similar process may take place in man also (76). A method as yet untested has been suggested for the histochemical detection of bilirubin (127).

*Kidney*. For obvious reasons, the conventional type of histochemical work on the mammalian kidney is not quantitative. Hence such studies as do exist are hardly more than suggestive in contrast with the extensive quantitative analyses of nephric function elaborated by Richards and his co-workers on amphibians (114). Ferrocyanide, uric acid, and hemoglobin were found to be eliminated by the glomerulus in a series of mammals (44, 34, 36). There was no evidence that tubular secretion plays a rôle in the elimination of the first two substances mentioned, though it does enter into the excretion of the last substance. The proximal convoluted tubule was shown to be of prime importance in the secretion of phenol red (35). In addition it was found that ferrocyanide is retained in some non-diffusible form in the cells of the proximal convolution weeks after it has ceased to be detectable in the urine. Except for these stored granules, ferrocyanide was not detected in any cells of the renal nephron. This observation was confirmed and used by Van Slyke, Hiller and Miller (134) in an evaluation of renal clearance methods. Their work forms a link in the chain of evidence which supports the use of inulin as a measure of glomerular filtration (126). A comparison of the relative density of ferrocyanide, uric acid, and hemoglobin in different parts of the lumen of the renal nephron led to the conclusion that water is reabsorbed in the descending limb of the loop of Henle. The use of ferrocyanide as an indicator of glomerular function and of phenol red as an indicator of secretory activity in the proximal convolution made possible an extensive study of the functional regression (in the mesonephros) and development (in the metanephros) of renal nephrons in early mammalian embryos of the cat, rabbit, pig and opossum and in the chick. It was found that both the mesonephros and metanephros in these early forms function simultaneously and continuously; urine is formed at a slower rate than in adults. Renal elimination was found to be a continuous function without any interruption due to degeneration of the mesonephros. The onset of tubular elimination of phenol red and of the reabsorption of water in the loop of Henle is accompanied by structural differentiation of these portions of the nephron. The onset of the glomerular elimination of ferrocyanide is not concomitant with any apparent renal differentiation—rather the inception of glomerular function appears to be correlated with extrarenal vascular phenomena. Structural and functional correlations were extended to an analysis of renal function in human embryos based on stained sections (37). Various aspects of these ontogenetical studies were confirmed by later work of Flexner and Gersh (27), Flexner (26), and of Cameron and Chambers (8).

## CONCLUSIONS

The last decade has witnessed the development of methods that have made possible histochemical studies on the elusive diffusible elements which occur normally in cells and in the tissue fluid and on complex organic substances present in cells or in their secretions, such as enzymes and nucleic acids. Most important is the trend toward the introduction of quantitative concepts. It is true that the results at hand are for the most part diffuse, rather superficially covering widely varied fields. The continued exploitation of concepts and methods in common use in the physical sciences should obviate this handicap by making possible quantitative histochemical estimations of substances present in concentrations as minute as they are in tissues.

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# INTERMEDIARY METABOLITES AND RESPIRATORY CATALYSIS

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Our knowledge and understanding of the mechanisms which bring about biological oxidation-reduction reactions have advanced rapidly in recent years. One result of this new knowledge is that the distinction between catalyst and substrate has in some respects become less sharp than previously. We may consider, for instance, the reversible oxidation of lactic acid to pyruvic acid by the system consisting of lactic apo-dehydrogenase, coenzyme I, a flavoprotein and methylene blue. The apo-dehydrogenase causes a transfer of hydrogen atoms between lactate (or pyruvate) and coenzyme I (or dihydro-coenzyme I). Coenzyme I is often called the prosthetic, or active, group of lactic dehydrogenase (i.e., of the holo-dehydrogenase), but it may also be regarded as being, like lactate, a substrate of the apo-dehydrogenase (see Dixon and Zerfas, 43). The protein part of the yellow enzyme causes a transfer of hydrogen between coenzyme and flavin nucleotide, the reduced form of the latter being reversibly oxidizable by methylene blue. The protein part of the yellow enzyme may be regarded as having two immediate substrates, namely, coenzyme I (or dihydro-coenzyme I) and the ribo-flavin-phosphate or flavin-adenine-dinucleotide. Only the fact that the flavin derivative has a high affinity for the specific protein, so that the two combined as a "yellow enzyme" were first isolated, has led us to think of the flavin nucleotide rather than the pyridine nucleotide (coenzyme I or II) as the active group of the "holo" yellow enzyme.

Catalysts for the processes of biological oxido-reductions thus fall into two apparently fundamentally different classes. There are the specific proteins, whose mechanism of action is unknown, and the hydrogen carriers, or electron transporters, like the pyridine and flavin nucleotides, whose rôle is well understood. The second class, the carriers, are, in their relations to the specific proteins, scarcely distinguishable from the metabolite substrates. Nevertheless, the pyridine, flavin, and possibly also the thiamine, derivatives serve physiologically purely as catalysts, mediating the oxidation and reduction of metabolites. But

work of recent years has shown that various substances long known only as intermediate products in the metabolic breakdown and synthesis of carbohydrates, proteins, and fats, also play a rôle as catalysts. In many cases the same oxidation-reduction reaction serves either as a step in the metabolism of the substance in question or as a catalytic mechanism in the metabolism of a different, or even of the same, substance. The whole situation illustrates the dynamic interlocking of processes in the living cell, and emphasizes the danger of oversimplification by too precise classification of processes.

In the following pages a number of processes which are both steps in catabolism and in the catalytic machinery will be discussed, reference being made chiefly to recent work on animal tissues.

One or two remarks on the condition of the material studied should first be made. There are essentially five types of tissue preparation commonly used in the type of work to be discussed, namely, the whole organism, the whole organ, slices, disintegrated whole tissue, and isolated or partially isolated systems. Work on whole animals and organs, the most physiological method, tends to give only over-all information, while work on isolated systems, the most analytical method, has provided most of our knowledge of enzymes, coenzymes and isolated reactions. It is work on the intermediate preparations, slices and "breis", which has provided most of the information here to be discussed. Slices approach the physiological condition of the tissue and make possible analytical and gasometric studies which help to throw light on what a tissue normally does. Disintegration of tissue allows various essential materials to be more readily diluted by the suspending medium or destroyed by enzymes, so that their concentration falls below the optimal and their addition produces striking effects which are often not found with slices. Disintegrated tissue therefore helps us to discover essential substances and reactions and to learn about the inter-relation between reactions. However, there is not always an absolute difference between the different methods and information of all kinds has been obtained with all the methods.

THE FOUR-CARBON DICARBOXYLIC ACIDS. a. *The $C_4$ dicarboxylic acids as intermediary metabolites.* The presence of these acids, succinic, fumaric, malic and oxaloacetic, in animal tissues has long been known. Toenniessen and Brinkman (145; see also Needham, 108) concluded from the results of perfusion experiments that these substances were intermediate products in a cycle of reactions through which lactate was oxidized to $CO_2$ and water in muscle. According to this scheme, two

molecules of pyruvate, formed by oxidation of lactate, underwent an oxidative condensation and hydrolysis yielding a molecule each of succinate and formate; diketo-adipic acid,

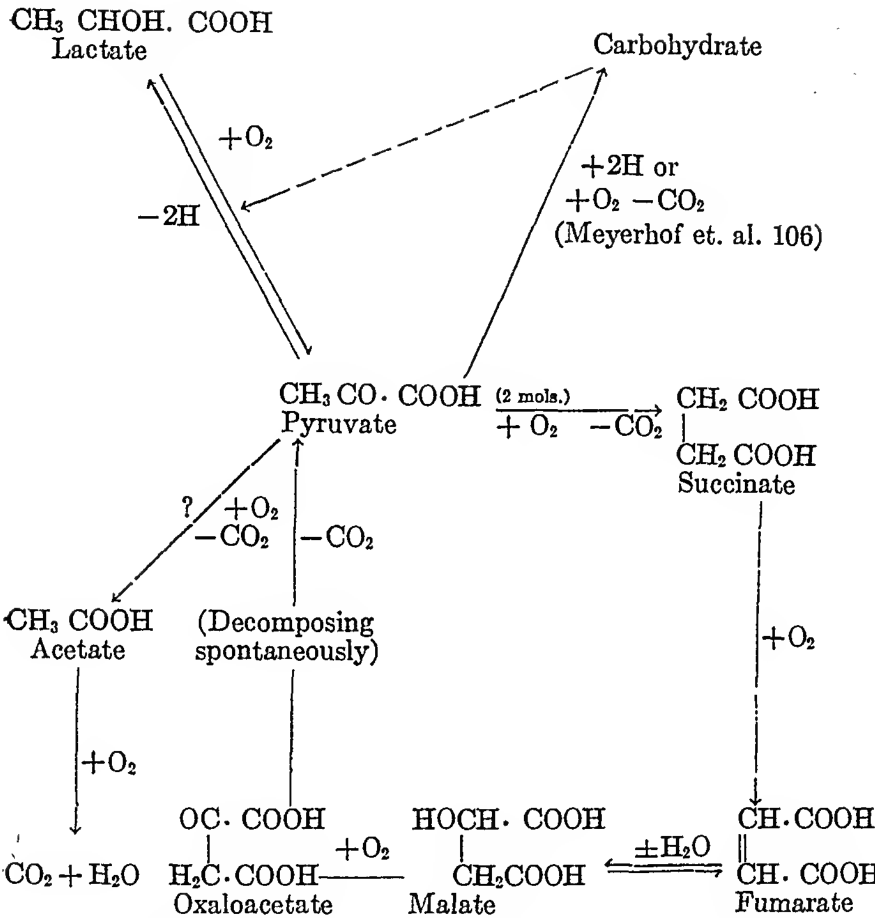
$$\begin{array}{l} CH_2CO \cdot COOH \\ | \\ CH_2CO \cdot COOH \end{array}$$

, was postulated as an intermediate in this transformation. The formate was oxidized away. The succinate was oxidized to fumarate which was converted by fumarase to malate and the latter oxidized to oxaloacetic acid which decomposed yielding $CO_2$ and one molecule of pyruvate in the place of the two molecules of pyruvate with which the cycle started.

In a series of studies of the metabolism of these compounds by sliced tissues, Elliott and co-workers (50, 45, 47, 49, 64, 23) showed clearly that the $C_4$-dicarboxylic acids can be intermediate products in the breakdown of pyruvate in kidney cortex, though with other tissues, rat liver, brain, testis, and transplantable tumors, ox retina, and chick embryo, this did not seem probable. Satisfactory muscle slices could not be prepared; the reactions in minced muscle and other tissues are discussed in detail later. For kidney cortex a number of transformations of pyruvate were indicated and a cycle similar to, but not identical with, that of Toennicssen and Brinkmann was postulated. (See diagram on p. 270.)

The occurrence of each step in the cycle involving the $C_4$ acids was proved. The kidney cortex slices caused a rapid disappearance of acid groups when lactate, pyruvate, or any of the $C_4$ compounds were added to the medium; the oxygen uptake was increased and the R.Q. changed in the direction to be expected if the added substance were being oxidized. With pyruvate and oxaloacetate, rapid removal of these bisulfite-binding substances took place. The formation of considerable amounts of succinate in the presence of added pyruvate, especially when malonate was also added to inhibit further oxidation of succinate, was demonstrated. Good evidence for the oxidation of succinate to fumarate, the equilibrium between fumarate and malate, and the oxidation of malate to oxaloacetate was obtained. Oxaloacetate decomposed spontaneously to pyruvate under the experimental conditions. Formate was not oxidized and it apparently played no part in the cycle.

The cycle did not account for the whole metabolism of pyruvate. It seemed probable that some acetate was formed and added acetate could be oxidized by kidney slices, though not as rapidly as pyruvate itself. More of pyruvate, lactate, and the $C_4$ compounds was used up than could be accounted for by complete oxidation, and a synthesis of




Transformations of Pyruvate in Kidney Cortex Slices

total carbohydrate was found (Benoy and Elliott, 23) in about the amount necessary to make up the balance sheet of pyruvate disappearance. The fact that succinate, fumarate, and malate, besides pyruvate, also gave carbohydrate synthesis, is added evidence for the cyclic relations of these substances. Of course, lactate, which was readily oxidized to pyruvate, also gave carbohydrate. (The carbohydrate formed appeared not to be glycogen.)

The cycle as given does not show all the steps in pyruvate breakdown. The intermediate steps between pyruvate and succinate were not known, and there are certain problems the solution of which may alter the whole picture. Malate was found to give rise to considerably

more succinate formation than did pyruvate. This could perhaps be accounted for by reduction of fumarate occurring as well as oxidation via pyruvate. The steps after malate require clarification. According to the above scheme, the complete oxidation of added malate depends upon the decarboxylation of oxaloacetate formed, but Elliott *et al.* found no catalyst for this non-oxidative step,[1] and, though added oxaloacetate decomposed rapidly enough to satisfy the scheme, the spontaneous process should be very slow in the low concentration of oxaloacetate which can be produced from malate (see p. 293). Kalekar (73) found that dialyzed kidney extracts took up oxygen rapidly when malate and phosphate were added and phosphopyruvate was formed, fluoride being present to inhibit the breakdown of the latter.

In view of the work on the rôle of citrate, to be discussed later, Greig and Elliott (unpublished results) tried adding citrate and its oxidation product, α-ketoglutarate, to kidney slices. These substances behaved very similarly to the other substances discussed in increasing respiration and being themselves used up. Citrate acid groups were removed considerably more slowly than malate acid groups; α-ketoglutarate was intermediate. This suggests that these substances may be outside the cycle in the same way as lactate is. On the other hand, definite traces of citrate were formed in an aerobic experiment with added pyruvate and somewhat more with oxaloacetate, (—allowing for a slight blank found with oxaloacetate without tissue. Adding pyruvate together with oxaloacetate gave no extra citrate.) Even if formation of citrate were a step in the cycle, a large accumulation of citrate would not be expected since added citrate was found to disappear as citric acid quite rapidly, probably by conversion into *cis*aconitate and *iso*citrate as well as by oxidation. Breusch (35) found a synthesis of citrate from oxaloacetate and pyruvate by minced kidney.

Anaerobic experiments with oxaloacetate gave no clue to its aerobic fate, the results being all accounted for by spontaneous decomposition, slight reduction to malate, slight reduction to lactate of the pyruvate formed on decomposition, and slight extra glycolysis stimulated by pyruvate. No citrate was formed. Aerobically, considerably more oxaloacetate was removed in the presence of tissue than in its absence, indicating an oxidative removal of oxaloacetate by kidney tissue, possibly an oxidative synthesis with some other molecule.

Sliced kidney cortex was the only tissue tried in which pyruvate seemed to be removed by successive transformations through the $C_4$ dicarboxylic acids. In all the other tissues tested, malate was either not oxidized away or it went too slowly to account for the observed pyruvate oxidation. Nor could formation of succinate from pyruvate in amounts

[1] Breusch (35), however, found a thermostable accelerator of oxaloacetate decarboxylation in minced tissues from all organs tried. The activity was apparently low. (See also Straub, 135.)

comparable with that given by kidney cortex be found, though small amounts were formed in other tissues (see also Weil-Malherbe, 149). Liver slices synthesized carbohydrate from added lactate and pyruvate but not from the other substances. Minced kidney did not behave like sliced kidney cortex.

In view of what follows, the point to be emphasized here is that in kidney cortex slices, pyruvate, succinate, fumarate, malate, oxaloacetate, $\alpha$-ketoglutarate, and citrate not only increase the rate of respiration but are themselves removed at a considerable rate. Though the oxygen uptake was considerably increased, the amount of acid disappearance could often be accounted for only by assuming that the entire respiration was diverted to oxidizing the added substance and that some of the acid had been synthesized to carbohydrate. There is no doubt that these added substances were not acting purely or mainly as catalysts in kidney cortex slices. With minced tissues there is evidence that the $C_4$ acids, especially when added in amounts larger than necessary to produce catalytic effects, can be oxidized away at an appreciable rate (see p. 278).

b. *The $C_4$ dicarboxylic acids as catalysts.*[2] Most of the work on this subject has been done with minced or finely disintegrated[3] tissue, particularly pigeon breast muscle, the latter material being chosen on account of its high respiratory activity and ready availability.

The evidence of cyanide inhibition and of spectroscopic studies has shown that most of the oxygen uptake of the cell takes place through the activity of the "respiratory enzyme" of Warburg, or cytochrome oxidase. This causes the oxidation of cytochrome (the various cytochromes being spoken of collectively) and the cytochrome is reduced again by other constituents of the cell. But succinate with its specific dehydrogenase was, until recently, the only dehydrogenase system known to reduce cytochrome. Succinic dehydrogenase is remarkably active in many tissues and Göszy and Szent-Györgyi (58) suggested that succinate-fumarate with the dehydrogenase acts as a link between other metabolites and the cytochrome-cytochrome oxidase system. (This latter system will be referred to as the WKS, the Warburg-Keilin System.) They suggested that the metabolites activated by their

[2] See Szent-Györgyi's review (142); for another review covering certain recent work in more detail, see Stare and Baumann (130).

[3] The Szent-Györgyi school passed the tissue through a Latapie mill from which the outer grinding disk had been removed so as to obtain the tissue in small pieces rather than completely disintegrated. Krebs and Eggleston (81), however, found that finer mincing made no significant difference.

dehydrogenases reduce fumarate to succinate which is then reoxidized by the WKS.

Szent-Györgyi and his co-workers (58, 7, 6) (see also Stare and Baumann, 129) found that the respiration of muscle suspensions was increased, or rather stabilized at the initial high rate, by the addition of a little fumarate, whereas it fell off otherwise, apparently as a result of loss by diffusion into the medium of fumarate originally present in the tissue. With added fumarate the R.Q. was maintained at the normal value of about 1.0 instead of falling off to a lower value. The added fumarate was not oxidized away. Addition of malonate, which is believed to be a specific[4] competitive inhibitor of succinic dehydrogenase (118), diminished the rate of respiration.

It was found, however, that the malonate inhibition could be overcome by adding fumarate. That is, with added fumarate, respiration continued in spite of the inhibition of succinate oxidation. Since this observation seemed to disprove the succinate-fumarate theory, it was suggested (7, 6) that the $C_4$ acids derived from fumarate could act as catalysts. Oxaloacetate behaved like fumarate in stabilizing the respiration,[5] reduction of added oxaloacetate to give fumarate and malate occurred very rapidly in the tissue, and oxidation of added fumarate to oxaloacetate could be detected when the oxaloacetate was fixed by hydrazine. From the muscle tissue a strongly active malate[6] dehydrogenase could be obtained. It was therefore suggested that the malate-oxaloacetate oxidation acted catalytically. This system does not react directly with the WKS but a thermolabile "Zwischensubstanz"[7] appeared to be present which acted as intermediate hydrogen transporter between malate[6]-oxaloacetate and the WKS.

An explanation of the effect of malonate + fumarate was later found, and by uniting the two theories (95, 141) all the observations could be fitted together and account taken of the wide distribution and high

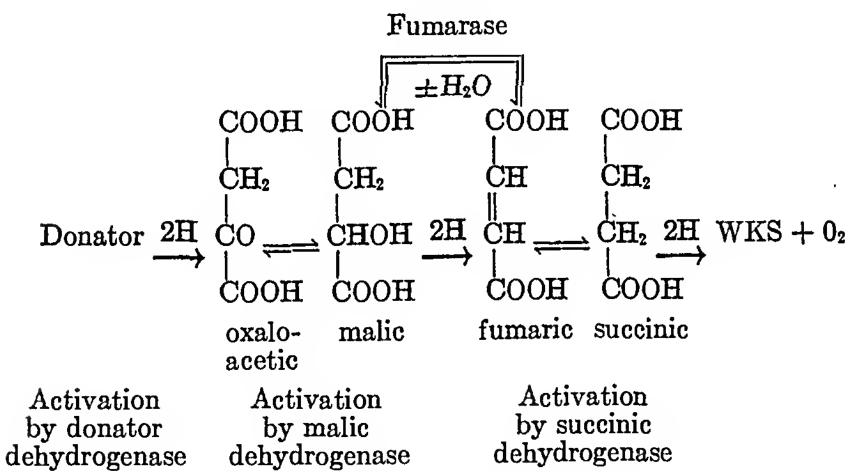
[4] Weil-Malherbe (149) indicated that malonate inhibits other systems, especially in high concentrations (above 0.02 M).

[5] When excess oxaloacetate was added, the $O_2$ uptake was suppressed for a few minutes until the oxaloacetate was reduced, apparently because oxaloacetate, even in low concentration, inhibits malate oxidation. The observation can be harmonized with the later united theory by Das' (40) observation that oxaloacetate is also a strong inhibitor of succinic dehydrogenase.

[6] At first it was believed that fumarate, not malate, was oxidized to oxaloacetate. See, however, Green (59), Laki (91).

[7] Though Szent-Györgyi (see p. 107 of (6)) later considered the evidence for the existence of the "Zwischensubstanz" to have been incorrect, such a catalyst, diaphorase or coenzyme factor, is now known to exist.

activity of the non-oxidizing enzyme, fumarase, which establishes an equilibrium between fumarate and malate. According to this theory the hydrogen from tissue donators, activated by the relevant enzymes, reduces oxaloacetate to malate under the influence of malic dehydrogenase. The malate, still activated by its dehydrogenase, is then reoxidized to oxaloacetate and the hydrogen is transferred to fumarate, activated by succinic dehydrogenase. Coenzyme I presumably mediates these hydrogen transfers. The succinate formed, still activated by its dehydrogenase, is reoxidized to fumarate by the WKS. Fumarase establishes an equilibrium between fumarate and malate maintaining the right proportions of these substances. The united theory may be represented by the following diagram in which the heavy arrows indicate transfers of hydrogen atoms from one substance to the next.




The Szent-Györgyi Scheme

Measurement of the redox potential of malate-oxaloacetate (Laki, 94) and experiments with isolated systems (Green, 59; Dewan and Green, 42) showed that such a series of oxidations was thermodynamically possible and the transfer of hydrogen from malate to cytochrome through fumarate-succinate was demonstrated (Straub, 136).

The malonate + fumarate effect, mentioned above, was explained by the Szent-Györgyi school as follows (40, 136). Malonate inhibits succinate oxidation because, while it is not itself oxidized, it is more readily adsorbed on the dehydrogenase than is succinate. But fumarate has a greater affinity for the enzyme than has malonate, so it can, to some extent, be adsorbed, activated, and reduced to succinate. The suc-

cinate formed does not in general leave the enzyme surface before it is reoxidized by the WKS. Some succinate must escape, and, being unable to compete with the malonate for return to the enzyme surface, it accumulates as the donator systems of the tissue reduce fresh molecules of fumarate. In this way fumarate is gradually removed from activity and inhibition of respiration sets in early if there is only the original trace of fumarate in the tissue, but no inhibition occurs for a long time if excess fumarate is added. This theory explained the observed accumulation of succinate when fumarate and malonate were present. An earlier explanation of this (6), later abandoned, was that fumarate (malate) was oxidized to oxaloacetate which then suffered an "over-reduction", accepting 4H atoms to give succinate directly by a mechanism unaffected by malonate. According to Krebs (see Citric Acid Cycle), succinate is formed from fumarate in the presence of malonate as a result of an oxidative series of reactions including a synthesis of citrate from oxaloacetate and pyruvate.

Banga (12; see also Greville, 63) fractionated ground muscle suspensions. She obtained a sediment containing the WKS, succinic and malic dehydrogenases, and other enzymes, and a fluid containing the thermostable donator, coenzymes, and an "activator". The "activator" was a protein which was separated by repeated acetone precipitation and appeared to be the dehydrogenase of the donator. The donator could be replaced by hexose mono- or di-phosphate, which is split prior to dehydrogenation into triosephosphate.[8] Washed enzyme suspension + a crude coenzyme preparation[9] + "activator" + hexosephosphate gave a system which respired strongly, especially if an extra trace of fumarate were added, but the oxygen uptake rate varied and usually was not equal to that of normal respiration. But on adding yellow enzyme (Warburg's first yellow enzyme from yeast) the oxygen uptake reached a very high rate. It was concluded that yellow enzyme was necessary to mediate the transfer of H from the malate-oxaloacetate system to the succinate-fumarate system and that traces of yellow enzyme already present in the mixture accounted for the activity when

[8] Banga and Szent-Györgyi (17) have shown that hexosediphosphate is rapidly split to triosephosphate by zymohexase in the sediment. Triosephosphate can replace hexosediphosphate in the system. The "activator" contains triosephosphate dehydrogenase and also a usually less active α-glycerophosphate dehydrogenase.

[9] Presumably the enzyme suspension or the coenzyme preparation contained Mg ions and other possibly necessary factors.

no extra yellow enzyme was added. Yellow enzyme has a redox potential between those of succinate-fumarate and malate oxaloacetate, it is reduced by the malic system, and Laki (93) showed that it is oxidized by fumarate with succinic dehydrogenase.

Straub (136) and Banga (13) have shown that $C_4$ substances mediate not only oxygen uptake via the WKS but also the reduction of methylene blue and other dyes by unwashed muscle, liver, or kidney tissues. If the succinic dehydrogenase was inhibited by malonate, or if a dye of redox potential too negative to be reduced by succinate but reducible by malate, was used, reduction still occurred but was slower.[10]

Banga found that malonate often had no effect on the rate of methylene blue reduction by well washed tissue + "activator" + coenzyme + hexosediphosphate. However, after dialysis of the tissue, addition of a trace of fumarate accelerated the reduction, and malonate inhibited it. It seeemed that, even after washing, traces of $C_4$ remained in the tissue (not enough for normal respiration) and these could only be removed by prolonged dialysis, and that the dye reduction, which is slower than the $O_2$ uptake, is limited not by the $C_4$ system but by other factors. Szent-Györgyi (141) believed that succinate-fumarate and malate-oxaloacetate are firmly held by the enzymes in a permanently activated condition, that in each case they were dealing not with a dehydrogenase and substrate but with a "transportase" consisting of a protein (the dehydrogenase) and a prosthetic group ($C_4$ compound). Like the prosthetic group of a yellow enzyme, $C_4$ can dissociate off. When excess of succinate, for instance, is present, the succinate will continually displace fumarate from the dehydrogenase and rapid oxidation of succinate to fumarate can occur.

The respiration of minced tissues and the effect of fumarate are considerably affected by the composition of the medium. Stare and Baumann (129; see also Greville, 63) found that the addition of heated muscle extracts, presumably containing coenzymes and donators, considerably increased (or maintained) the respiration of minced pigeon muscle and enhanced the effect of fumarate. While the respiration rate per unit weight falls off rapidly on diluting tissue suspensions, Krebs and Eggleston (81) found that addition of muscle extract prevented this fall. With several tissues, Straub and Annau (137) had

[10] With a dye of such negative redox potential the rate of decolorization was *accelerated* by malonate. The fumarate formed by reduction of oxaloacetate evidently accepted H from the reduced dye, i.e., reoxidized it, unless the fumarate-succinate system was inhibited by malonate.

found fumarate effects less marked in Ringer solution than in plain phosphate buffer, but this was probably due to the fact that the Ringer solution contained Ca, whereas the phosphate buffer did not. Ca, in very low concentration, inhibits the respiration and fumarate effect with minced tissues[11] (Greville, 62; Krebs and Eggleston, 81; Elliott and Elliott, 46). Stare and Baumann (129) (see also Krebs and Eggleston 82) found higher respiration rates and stronger fumarate effects in Ringer-phosphate solution (Ca-free) than in plain phosphate buffer. With disintegrated liver Elliott and Elliott (46) found that the respiration was low and added fumarate had no effect unless an adequate concentration of univalent anion (normally chloride) was present in the medium. The effects of NaCl and malate only occurred when the suspension of tissue was fairly concentrated, about 1:10 on wet weight. With more dilute suspensions, other components of the respiratory system were apparenly diluted to less than the optimum concentration. Greville (63; see also 51) found that Mg ions are necessary for the proper respiration of minced pigeon muscle.

Most of the work on this subject has been done with pigeon muscle. The main observations have been confirmed with liver and kidney by Annau (4), Banga (11), Stare (128), and other workers. Malonate inhibits the respiration of minced liver and kidney, and small amounts of added fumarate cause large increases in, and stabilization of, the respiration rate of these tissues. Fumarate is oxidized to oxaloacetate and added oxaloacetate is rapidly reduced to malate. Liver and kidney suspension behaved similarly to pigeon muscle in the reduction of dyes (Straub, 136). Greville (62) showed fumarate and malonate effects with rat diaphragm and sliced brain, and Banga et al. (14) showed that brei or fine dispersion of pigeon brain rapidly reduces oxaloacetate. The respiration of various minced muscles other than pigeon breast,—chicken breast, pig and rabbit heart, rabbit, rat and human skeletal muscle (Stare and Baumann, 129, 130),—have been shown to be increased by the addition of fumarate (or citrate) especially in the presence of boiled muscle juice. Fumarate (or citrate) increased the respiration of Flexner-Jobling sarcoma slices somewhat in the presence of boiled muscle juice; no effect was observed with mince or in the absence of muscle juice. Boyland and Boyland (30) found slight effects of fumarate and malonate with Jensen and Crocker sarcoma. Nitro-

[11] Ca has no effect on the respiration of intact diaphragm (62) or on sliced liver or kidney (81, 46).

phenols increase tissue respiration and Greville indicated that the extra respiration also goes through the fumarate system.

Szent-Györgyi (141) considers it probable that not all metabolites are oxidized through the whole $C_4$ system. Some may reduce fumarate to succinate directly without the mediation of oxaloacetate-malate. Lactate, for instance, has about the same redox potential as malate and so would not be expeeted to reduee oxaloaeetate.

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[12] Lipmann (97) found that "protein" preparations from *B. delbrückii* required, for the oxidation of pyruvate, cocarboxylase, Mn or Mg or Co, and phosphate, and also flavin-adenine-dinucleotide, but did not require added $C_4$ substances.

and Green (42), with isolated enzyme systems, showed the oxidation of β-hydroxybutyrate by fumarate. Annau (4, 5) showed by qualitative tests that small amounts of acetone bodies were formed by liver slices or mince without addition, and considerably more when pyruvate was added. The inhibitor, malonate, increased the accumulation of acetone bodies and the addition of fumarate diminished it. Kidney also formed acetoacetate in the presence of pyruvate and malonate. Elliott and Elliott confirmed quantitatively a rapid accumulation of acetoacetate from pyruvate with liver (only in the presence of NaCl), and decreased amounts when a little malate or oxaloacetate was present. Some acetoacetate was also formed from lactate and citrate. Annau concluded that fumarate catalyzed normal oxidation of pyruvate, and in its absence acetoacetate was formed by less normal condensation and oxidation; but Elliott suggested that fumarate catalyzes the oxidation of acetoacetate.

What proportion of the normal respiration of various tissues is carried through the $C_4$ system is not decided. Most normal tissues contain enough succinic dehydrogenase and WKS to account for the whole normal respiration through the $C_4$ system (Elliott and Greig, 48; see also discussion in 64). Malic dehydrogenase was present in all tissues tested by Green (59). On the other hand, chick embryo, thymus, spleen, pancreas, and certain rat tumors are deficient in succinic dehydrogenase (45, 48, 64). Breusch (35) found strong oxaloacetate reduction with minced muscle, liver and kidney, some reduction with brain and pancreas, but negligible oxaloacetate (or pyruvate) reduction with spleen, lung, placenta and peripheral nerves. Also certain rat tumors (Banga, 11) and embryo tissue (Blaszó, 26) reduce oxaloacetate or pyruvate scarcely at all.

Quastel *et al.* (119) had shown that *B. coli* could grow anaerobically with glycerol or lactate plus fumarate in the medium, the fumarate serving as H acceptor for the oxidation of the substrates. Krebs (76) produced evidence that, in *B. coli*, fumarate catalyzes the aerobic oxidation of glucose, malate, lactate, acetate, glycerol, glyceraldehyde, butyrate, pyruvate, acetoacetate, *l*(+)-glutamate, and molecular hydrogen. The oxidation of lactate, malate, and of formate could take place independently of fumarate but most of the aerobic oxidation of the other substances seemed to be mediated by fumarate-succinate. Oxaloacetate also appeared to be a hydrogen carrier in *B. coli*.

It is now known that diaphorase, coenzyme factor, makes possible the transfer of hydrogen from donators, activated by coenzyme-determined dehydrogenases, directly to the WKS without the mediation of

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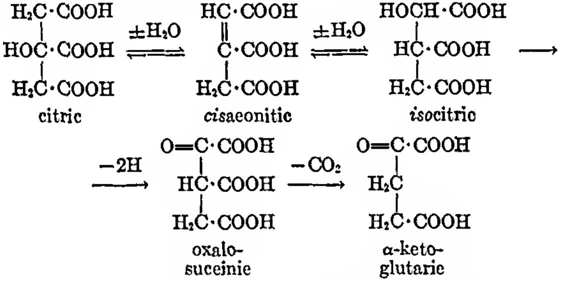
tarate when fed the $C_4$ dibasic acids, or pyruvate, α-ketoglutarate, β-hydroxybutyrate, acetoacetate, or various other aliphatic acids. With humans, oral administration of large doses of sodium pyruvate gave rise to the excretion of large amounts of α-ketoglutarate; the citrate excretion was raised also, but not more than by the administration of equivalent amounts of sodium bicarbonate. With rats, vitamin B deficiency caused a raised excretion of α-ketoglutarate which was reduced on administration of vitamin $B_1$ (Simola, 123).

That mammalian and other tissues can oxidize citrate rapidly has been known since the work of Battelli and Stern. Bernheim (24) described an active enzyme preparation from liver, and, using this preparation, Martius (104) showed that the main course of citrate oxidation probably occurs as follows:

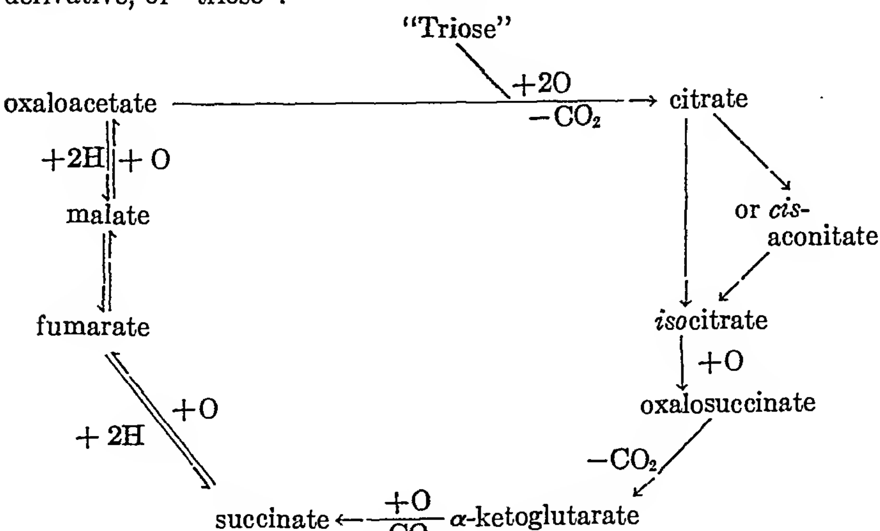



The citrate is converted non-oxidatively to *cis*aconitate and *iso*citrate by an enzyme similar to fumarase, and the *iso*citrate is then dehydrogenated by an enzyme which is now known to be a coenzyme-determined dehydrogenase (2). The first oxidation product is probably oxalosuccinate which breaks down to give α-ketoglutarate. In the presence of other enzymes the α-ketoglutarate can be oxidized and decarboxylated to give succinate. The equilibrium between citrate, *cis*aconitate, and *iso*citrate, and the oxidation to α-ketoglutarate have been confirmed by other workers (34, 71).

As was mentioned on page 271, Elliott and Greig found that kidney cortex slices are able rapidly to oxidize citrate and α-ketoglutarate away. The formation of citrate from various substrates and combinations of substrates, and the oxidation of citrate and α-ketoglutarate, with minced tissues are discussed below.

b. *The citric acid cycle of Krebs.* Krebs and his co-workers (84, 81)

found that added citrate or *iso*citrate behaved similarly to the $C_4$ dicarboxylic acids in catalytically promoting the respiration of pigeon muscle suspensions, especially if glycogen, hexosediphosphate, or $\alpha$-glycerophosphate were also present. Since citrate promoted respiration catalytically—that is, caused a greater $O_2$ uptake than its own oxidation could account for,—Krebs and Johnson believed that citrate was oxidized and regenerated. When arsenite or malonate was added, it was found that citrate disappeared. Arsenite inhibits the oxidation of $\alpha$-keto acids and, in the presence of arsenite, accumulation of $\alpha$-ketoglutarate in the expected amount was found. With malonate to inhibit succinate oxidation, the expected amount of succinate was found. Finally, on incubating oxaloacetate with minced muscle anaerobically, they found a synthesis of citrate. The two additional C atoms were presumed to have come from carbohydrate derivatives. (Martius and Knoop (105) had found that citric acid is readily formed without tissue when oxaloacetate and pyruvate together are treated with hydrogen peroxide in alkaline medium.) Though the synthesis involves oxidation, anaerobic conditions were necessary to prevent further oxidation of the citrate; the oxidation was believed to take place at the expense of reduction of other molecules of oxaloacetate to malate. Krebs believed, therefore, that a cycle of reactions takes place according to the following diagram in which the arrows indicate conversion of one substance to the next. The net effect is the complete oxidation of the carbohydrate derivative, or "triose".




Krebs' citric acid cycle

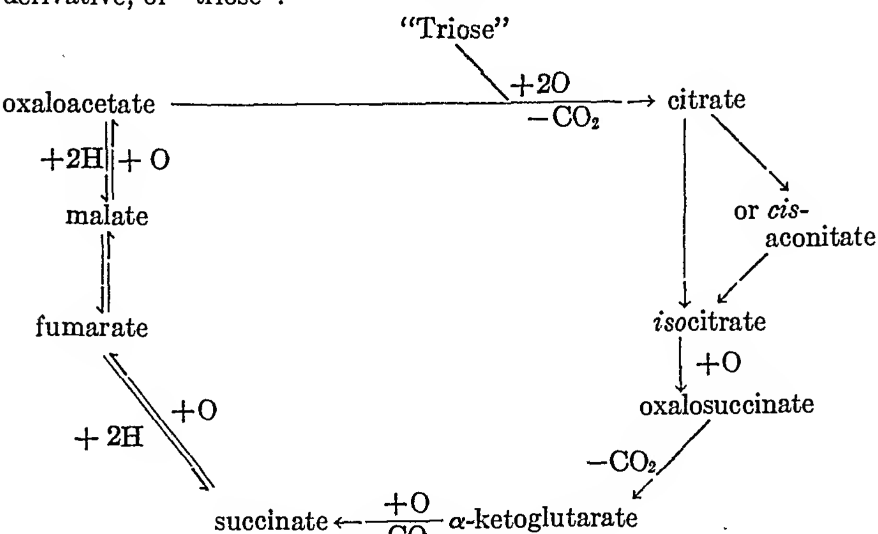
According to this scheme, succinate can arise from added oxaloacetate both by oxidation via citrate and by reduction via malate. As was therefore to be expected, they found that, aerobically, added malonate increased the yield of succinate from oxaloacetate by preventing further oxidation, while anaerobically malonate decreased the yield of succinate by interfering with fumarate reduction. All the substances of the citric acid cycle were equivalent in maintaining the respiration of muscle mince.

Krebs, Salvin, and Johnson (87) considered that the theory was supported by the observations mentioned above that injection of the salts of the $C_4$ dicarboxylic acids gave rise to increased excretion of citric and α-ketoglutaric acids. The fact that malonate had similar effects was explained as due to inhibition of citrate breakdown. Malonate is not itself metabolized but inhibits succinate oxidation, and as was expected, when malonate was administered, succinate was found in large amounts in the urine.

Some of Krebs' experimental results have been confirmed by other workers, but his results and conclusions have also been subjected to considerable criticism.

Breusch (34) and others observed the increase of respiration by citrate, *cis*aconitate, *iso*citrate, and α-ketoglutarate, and Baumann and Stare (22) found that the $C_4$ dicarboxylic acids, citrate, α-ketoglutarate, and glutamate (which is presumably converted to α-ketoglutarate by transamination), all caused total increases in the oxygen uptake of pigeon muscle greater than could be accounted for by simple oxidation of the added substances. Also with other muscle tissues, and with minced or sliced kidney (only in the presence of glucose) or liver, citrate (and fumarate) caused more sustained respiration, though the increase in oxygen uptake was not always sufficient to prove catalysis (130). But citrate was usually not as effective as fumarate; and Stare and Baumann (130) noted that higher concentrations of citrate (0.01–0.02 M) often inhibited respiration somewhat. Tables of Krebs and and Eggleston (81), and Long *et al.* (101), also show this effect. Thomas (144) and Stadie *et al.* (127) did not obtain sufficiently increased oxygen uptakes with pigeon muscle and citrate to prove a catalytic effect of the latter. Thomas found that the effect of citrate was small and was apparent only after a latent period whereas the effects of the $C_4$ acids were immediate. Krebs (78) points out that, when Thomas found no accelerating effect of citrate, his figures also did not show the well-confirmed catalytic effect of the $C_4$ compounds. Krebs also believes

found that added citrate or *iso*citrate behaved similarly to the $C_4$ dicarboxylic acids in catalytically promoting the respiration of pigeon muscle suspensions, especially if glycogen, hexosediphosphate, or $\alpha$-glycerophosphate were also present. Since citrate promoted respiration catalytically—that is, caused a greater $O_2$ uptake than its own oxidation could account for,—Krebs and Johnson believed that citrate was oxidized and regenerated. When arsenite or malonate was added, it was found that citrate disappeared. Arsenite inhibits the oxidation of $\alpha$-keto acids and, in the presence of arsenite, accumulation of $\alpha$-ketoglutarate in the expected amount was found. With malonate to inhibit succinate oxidation, the expected amount of succinate was found. Finally, on incubating oxaloacetate with minced muscle anaerobically, they found a synthesis of citrate. The two additional C atoms were presumed to have come from carbohydrate derivatives. (Martius and Knoop (105) had found that citric acid is readily formed without tissue when oxaloacetate and pyruvate together are treated with hydrogen peroxide in alkaline medium.) Though the synthesis involves oxidation, anaerobic conditions were necessary to prevent further oxidation of the citrate; the oxidation was believed to take place at the expense of reduction of other molecules of oxaloacetate to malate. Krebs believed, therefore, that a cycle of reactions takes place according to the following diagram in which the arrows indicate conversion of one substance to the next. The net effect is the complete oxidation of the carbohydrate derivative, or "triose".




Krebs' citric acid cycle

According to this scheme, succinate can arise from added oxaloacetate both by oxidation via citrate and by reduction via malate. As was therefore to be expected, they found that, aerobically, added malonate increased the yield of succinate from oxaloacetate by preventing further oxidation, while anaerobically malonate decreased the yield of succinate by interfering with fumarate reduction. All the substances of the citric acid cycle were equivalent in maintaining the respiration of muscle mince.

Krebs, Salvin, and Johnson (87) considered that the theory was supported by the observations mentioned above that injection of the salts of the $C_4$ dicarboxylic acids gave rise to increased excretion of citric and $\alpha$-ketoglutaric acids. The fact that malonate had similar effects was explained as due to inhibition of citrate breakdown. Malonate is not itself metabolized but inhibits succinate oxidation, and as was expected, when malonate was administered, succinate was found in large amounts in tho urine.

Some of Krebs' experimental results have been confirmed by other workers, but his results and conclusions have also been subjected to considerable criticism.

Breusch (34) and others observed the increase of respiration by citrate, *cis*aconitate, *iso*citrate, and $\alpha$-ketoglutarate, and Baumann and Stare (22) found that the $C_4$ dicarboxylic acids, citrate, $\alpha$-ketoglutarate, and glutamate (which is presumably converted to $\alpha$-ketoglutarate by transamination), all caused total increases in the oxygen uptako of pigeon muscle greater than could be accounted for by simple oxidation of the added substances. Also with other muscle tissues, and with minced or sliced kidney (only in the presence of glucose) or liver, citrate (and fumarate) caused more sustained respiration, though the increase in oxygen uptake was not always sufficient to prove catalysis (130). But citrate was usually not as effective as fumarate; and Stare and Baumann (130) noted that higher concentrations of citrate (0.01–0.02 M) often inhibited respiration somewhat. Tables of Krebs and and Eggleston (81), and Long *et al.* (101), also show this effect. Thomas (144) and Stadie *et al.* (127) did not obtain sufficiently increased oxygen uptakes with pigeon muscle and citrate to prove a catalytic effect of the latter. Thomas found that the effect of citrate was small and was apparent only after a latent period whereas the effects of the $C_4$ acids were immediate. Krebs (78) points out that, when Thomas found no accelerating effect of citrate, his figures also did not show the well-confirmed catalytic effect of the $C_4$ compounds. Krebs also believes

that many of the criticisms of Thomas and Breusch (see below) are not serious since their figures for normal oxygen uptake were lower than his, indicating that the experiments were made with tissue which had lost much of its metabolic activity. Krebs and Eggleston (81) had found effects of citrate and α-ketoglutarate as immediately apparent as those of the $C_4$ compounds; Elliott and Elliott found the same with liver. However, the effect of citrate does seem to be variable.[13]

Breusch (34) was unable to confirm the synthesis of citrate from oxaloacetate and tissue metabolites in pigeon muscle. He indicated that citrate was only found when abnormally high concentrations of oxaloacetate were used and that it arose artificially from a condensation product which is formed when oxaloacetate is neutralized in strong solution. Later he (35) found that minced kidney (aerobically or anaerobically) causes a condensation of oxaloacetate with pyruvate, glycerate, or dihydroxyacetone to give citrate. Minced liver, brain, and lung showed slight synthesis under anaerobic conditions, but he still found no citrate formation by muscle (cat or pigeon). No citrate was formed by kidney mince when the other $C_4$ substances, or α-ketoglutarate, glutamate, or various other substances were added instead of oxaloacetate. The formation of citrate from oxaloacetate by kidney was found to compete unfavorably with the reduction to malate. Citrate formation appeared to occur only above a certain threshold of oxaloacetate concentration and Breusch suggested that citrate formation was chiefly an elimination process for the disposal of surplus $C_4$ dicarboxylic acids, these not being excretable in the urine but convertible via oxaloacetate into citrate which can be excreted. Smith and Orten (126), as a result of citrate determinations in blood and tissues in normal and nephrectomized animals, had concluded that citrate production occurred chiefly in the kidney or was dependent on the presence of the kidney.

Breusch's claims that, with muscle, *a*, citrate arose from an impurity of oxaloacetate, and *b*, was found only with unphysiologically high concentrations of oxaloacetate, and *c*, in low amount, were answered by Krebs (78, 82) with the observations that in aerobic experiments, in the presence of excess pyruvate which suppresses the oxidation of other

[13] Krebs and Eggleston (81) stated that catalytic effects with the $C_4$ acids and citrate were not always found at pH 7.4 but regularly at pH 6.8. This statement seems to be contradicted in the same paper (table VII). Stadie *et al.* worked at pH 6.8 but did not obtain high enough increases in oxygen uptake to prove catalysis. In his later papers (82, 78) Krebs apparently worked at pH 7.4.

substances (see below), *a*, fumarate also gave citrate, and *b*, oxaloacetate would only be formed gradually by oxidation of fumarate, and that *c*, yields of 15 per cent of the maximum citrate were obtained. Higher yields of citrate were not to be expected since citrate is rapidly further oxidized and citrate removal is accelerated by oxaloacetate;—citrate is oxidized by oxaloacetate in the Szent-Györgyi manner (Krebs 79, see below). Simola and co-workers (65) found appreciable production of citrate by minced tissues from various organs with various substrates. With minced ox heart and brain, under aerobic conditions, citrate was found to be produced especially strongly in the presence of pyruvate plus fumarate, malate, or oxaloacetate. Hallman and Simola (66) consider that, at least in heart muscle, which can decompose citrate rapidly, pyruvate is decomposed by the citric acid cycle.

Breusch (34) and Thomas (144) found that citrate added to muscle tissue disappeared rapidly, even in the absence of arsenite or malonate, whereas regeneration through the cycle should maintain its concentration. But later Breusch (35) criticized the cycle theory with the observations that, while liver and kidney mince could break down citrate, muscle scarcely had any such activity. Krebs (78) pointed out that earlier workers had shown an active citrate oxidizing system in muscle and he calculated from Breusch's earlier figures that the citrate oxidation rate was more than sufficient to account for the total respiration rate of the tissue. Krebs believed that the oxidation of citrate can be demonstrated when the physiological balance is upset, as by adding excess citrate or an inhibitor of the cycle, otherwise regeneration via the cycle can prevent the observation of citrate oxidation.

Breusch (34) considered that, according to the cycle, more malate should be produced from added citrate than from oxaloacetate, but the reverse was found to occur. Thomas (144) found the formation of malate from added oxaloacetate was as rapid under anaerobic as under aerobic conditions, whereas the presence of oxygen would be required if the malate was to be formed by the citric acid cycle. The yield of malate was not diminished by the presence of malonate; malonate, by inhibiting succinate oxidation should decrease malate formation via the cycle. Krebs (84, 78), however, has always agreed that the reactions among the $C_4$ acids are readily reversible and, as will be shown below, reduction of oxaloacetate to malate plays an important part in the later more developed scheme of Krebs (79).

Breusch (34) found that addition of citrate was much less effective than fumarate in overcoming the malonate inhibition of respiration.

Baumann and Stare (22, 131) found the same thing with citrate and glutamate, though α-ketoglutarate was as effective as the $C_4$ substances. The reason for this was not clear, but since citrate did not behave like the other substances in this respect, it apparently could not be an essential part of a catalytic cycle with the $C_4$ substances.

Thomas (144) found that, in the presence of arsenite and dinitrophenylhydrazone, added malate, fumarate and succinate gave rise to the rapid formation of the pyruvic hydrazone, indicating a rapid formation from the $C_4$ substances of oxaloacetate, which broke down to pyruvate. But citrate did not give oxaloacetate (i.e., the pyruvic hydrazone) rapidly. Szent-Györgyi believes that arsenite inhibits the reducing donator systems and these results were interpreted to mean that, with the reducing systems inhibited, the $C_4$ acids but not citric acid are oxidized to oxaloacetate. If Krebs' citrate theory and his belief that arsenite inhibits the oxidation of α-ketoglutaric acid (or any α-keto-acid) were correct, α-ketoglutarate and not oxaloacetate should have accumulated from the added $C_4$ acids.

With liver suspensions, Elliott and Elliott (46) found that the respiration (in the presence of NaCl in the proper concentration) was approximately equally accelerated by lactate and pyruvate as well as by citrate and the other members of Krebs' cycle. They suggested that added lactate, pyruvate, citrate, and α-ketoglutarate are oxidized, yielding the $C_4$ substances, and that the latter act as catalysts in the manner suggested by Szent-Györgyi; Breusch (34) has made the same suggestion regarding the effect of citrate with pigeon muscle.

With dialyzed pigeon brain dispersions, in the presence of coenzymes and other additions, Banga, Ochoa, and Peters (14) found that the $C_4$ dicarboxylic acids were active in promoting pyruvate oxidation but citric and α-ketoglutaric acids were much less active. Citrate itself was not oxidized by the dialyzed dispersions with all the additions, nor had it been found to be oxidized by brain brei (101, 35). Nevertheless, brain does contain the citric (*iso*citric) dehydrogenase system (2, 71), and, as mentioned above, Breusch (35) and Simola (65) have found some citrate synthesis in brain.

The last few points against the citric acid theory have not yet been experimentally answered but suggestions concerning some of them are made in section *d*. Krebs (78) emphasizes the oxidative formation of succinate from oxaloacetate as conclusive evidence for the cycle. In the presence of malonate and low concentrations of oxaloacetate or

fumarate, pigeon muscle produced six to seven times more succinate aerobically than by anaerobic reduction.

Krebs has recently brought forward new evidence supporting and elaborating his theory.

Krebs and Eggleston (82) found that minced pigeon breast muscle in Ringer-phosphate medium, caused the rapid disappearance of added pyruvate, the rate falling off almost completely in the third or fourth hour. Some increase in the rate of oxygen uptake occurred, especially after about an hour. The total oxygen uptake, and the R.Q. found, corresponded approximately to complete oxidation of the pyruvate utilized and indicated that the oxidation of all other substrates had been suppressed. Malonate in high concentration (0.025M) inhibited the aerobic utilization of pyruvate by 92 per cent, the remaining 8 per cent being due to anaerobic processes unaffected by malonate. In low concentration (0.001M) malonate caused only 77 per cent inhibition. The addition of fumarate completely abolished the effect of low malonate but with higher malonate concentration fumarate caused a pyruvate utilization approximately equivalent to the amount of fumarate added. An additional uptake of 2 to 2.5 mols. of $O_2$ per mol. of fumarate added, occurred in the presence of high malonate (whether pyruvate was present or not, see below); 80 to 100 per cent equivalent of fumarate was recovered as succinate, and about 3 mols. of $CO_2$ were given off. An anaerobic experiment showed that the malonate effectively prevented any appreciable reduction of fumarate to succinate, so it was concluded that the succinate arose by an oxidative process according to equation (1).

$$\text{Fumarate} + \text{pyruvate} + 2O_2 = \text{succinate} + 3CO_2 + H_2O \quad (1)$$

Deviations from the theoretical values were attributed to some oxidation of fumarate to oxaloacetate, the latter decomposing to pyruvate and $CO_2$, some oxidation of lactate to pyruvate, and slightly incomplete inhibition of succinate oxidation. It was believed that when the malonate concentration was low, some oxidation of succinate through pyruvate could occur and reaction (1) would be repeated.

Equation (1) was believed to be the net effect of the following series of reactions:

$$\text{fumarate} \xrightarrow{+O} \text{oxaloacetate} \xrightarrow{\text{pyruvate} + O} \text{citrate} + CO_2$$

$$\xrightarrow{+O} \alpha\text{-ketoglutarate} + CO_2 \xrightarrow{+O} \text{succinate} + CO_2$$

Supporting this belief was the observation that approximately the expected amounts of oxygen uptake and succinate formation occurred, in the presence of malonate, with oxaloacetate + pyruvate, with citrate, or with α-ketoglutarate,[14] but not with a number of other possible intermediates. With fumarate and excess of pyruvate, yields of 50 per cent of the maximum possible amount of α-ketoglutarate and 15 per cent of citrate[15] were recovered; presumably the excess pyruvate, being preferentially utilized, suppressed further oxidation of these substances. Even without added pyruvate, some succinate and α-ketoglutarate were formed, the necessary pyruvate being formed from tissue carbohydrate.

The fact, mentioned above, that in the presence of high malonate even without added pyruvate, about 2.5 mols. of extra oxygen were taken up per mol. of fumarate added, and most of the fumarate was recovered as succinate, was taken to indicate that the oxidation of normal substrates behaves as pyruvate oxidation, and that the above reaction series represents the main steps in the bulk of the normal respiration in muscle, in which carbohydrate is known to be the chief substrate.

Very recently Krebs (79) has brought forward evidence which largely harmonizes his theory with that of Szent-Györgyi. In his opinion, the Szent-Györgyi theory of oxaloacetate $\rightleftharpoons$ malate serving as a hydrogen carrier, and the citric acid cycle as a series of steps in intermediary metabolism, are complementary processes. In the oxidation of carbohydrate by pigeon muscle, the first oxidative step is the oxidation of "triose" to pyruvic acid. (Presumably diphospho-glyceraldehyde is the substance actually oxidized,—Negelein and Bromel, 110; Warburg and Christian, 148.) As was shown by the Szent-Györgyi school (6,

[14] Krebs' table gives only $O_2$ uptake with α-ketoglutarate since succinate could not be determined in the presence of α-ketoglutarate and malonate. No data can be found to show directly the rapid formation of succinate from α-ketoglutarate in muscle. But there is ample evidence that citrate (*iso*citrate) is oxidized to α-ketoglutarate, that succinate can be produced from citrate, and that α-ketoglutarate shows effects on respiration similar to those of citrate and succinate. It is, therefore, inferred that citrate is oxidized to succinate via α-ketoglutarate. The formation of succinate in other tissues by dismutation of α-ketoglutarate with itself, or with acetoacetate, or with α-iminoglutarate is mentioned on pages 295 and 299.

[15] Krebs' figures for "citrate" include citrate, *cis*aconitate, and *iso*citrate, these substances being rapidly interconvertible in tissue, without any oxidation involved, and give an equilibrium mixture containing about 80 per cent citrate, 4 per cent *cis*aconitate, and 16 per cent *iso*citrate (Johnson, 71).

see also Parnas and Szankowski, 115), oxaloacetate accepts the hydrogen, yielding malate which can be reoxidized. Then pyruvate + oxaloacetate undergo oxidative synthesis to citric acid, and the hydrogen equivalent of the oxidation is, according to Krebs and Johnson (84), accepted by another molecule of oxaloacetate. The citrate formed is oxidized to $\alpha$-ketoglutarate, oxaloacetate again accepting the hydrogen. Krebs established this last point by finding that, anaerobically, added citrate alone was not removed, but it was rapidly removed when oxaloacetate was added, and an approximately equivalent amount of $\alpha$-ketoglutarate was formed; the rate was much greater than was necessary to account for the total respiration through the citric acid cycle. Krebs believed that oxaloacetate might be the H acceptor for the oxidation of $\alpha$-ketoglutarate to succinate, but he was unable to demonstrate this.[16] Thus oxaloacetate accepts three and possibly four out of six pairs of hydrogen atoms in the oxidation via the cycle of a molecule of triose. Of course all these hydrogen transfers are mediated by the coenzymes[17] under the influence of dehydrogenases. The malate in each case is reoxidized to oxaloacetate, the hydrogen being transferred to the cytochrome system and oxygen through coenzyme I, either via diaphorase or possibly through fumarate $\rightleftharpoons$ succinate.

The scheme in its present form is by no means the whole story of carbohydrate oxidation since details concerning the synthesis of citrate and the rôle of phosphorylations have not yet been worked out. The "triose" which is oxidized is a phosphorylated compound. With kidney extracts, Kalckar (73) observed the formation of phosphopyruvate when malate was being oxidized and Ferdmann and Epstein (55) report formation of phosphopyruvate when lactate is oxidized by cat muscle. Colowick, Welch and Cori (38) have found that a phosphorylation of glucose is linked with the oxidation of succinate. Lipmann (98, 99, see however 113) has suggested that, at least in *B. delbrückii*, the oxidation of pyruvate is actually an oxidation of a pyruvate-phosphate compound to acetylphosphate, the latter being able to transfer phosphate to adenylic acid leaving free acetic acid. Ochoa (112) finds that oxidation of pyruvate by brain dispersions is coupled with phosphorylation of adenylic acid.

c. *Insulin effects.* Krebs and Eggleston (81) found that in the pres-

[16] See footnote 14 on page 288.

[17] Krebs points out that *iso*citric dehydrogenase uses coenzyme II, while only dihydro-coenzyme I reduces oxaloacetate to malate; the mechanism of the reaction between citrate and oxaloacetate is therefore not yet clear.

ence of boiled muscle juice (see p. 276) and substances of the citric acid cycle, the addition of small amounts of insulin caused a further large increase in the respiration of minced pigeon muscle. The effect, which was most marked with citrate, appeared more as a stabilization of the inital high rate over a longer period than as an actual increase in the initial respiration rate.

This effect has been confirmed in principle by Shorr and Barker (122), Stadie, Zapp, and Lukens (127), and Stare and Baumann (131) though the effects found by these authors were smaller than those found by Krebs and Eggleston. Shorr and Barker found no insulin effect with any tissue other than minced pigeon muscle (chicken breast muscle, skeletal muscle of cat, dog, and rabbit, heart muscle of dog, and sliced pigeon muscle), though the other tissues all gave increased respiration when citrate and muscle juice were added. But Stare and Baumann (130), using Ringer-phosphate instead of plain phosphate buffer as medium, were able to produce some effects with chicken breast and rabbit heart and skeletal muscle. Stadie *et al.* did not find any increased activity of insulin *in vitro* with muscle from recently depancreatized pigeons or cats, but Stare and Baumann found the effect of insulin increased two or three times with muscle from pigeons depancreatized 1 to 3 weeks previously.[18] Stare and Baumann (131) found that the R.Q of minced pigeon muscle without additions fell from its initial high level to zero in the third or fourth hour of an experiment (the respiration also fell off greatly), while with insulin added, with or without added muscle juice and citrate or fumarate, the R.Q. was maintained near 1.0. Stadie *et al.* found the effect of insulin was no greater in the presence of citrate than in its absence; but Stare and Baumann, like Krebs and Eggleston, found that the effect of insulin, over the third and fourth hour of experiments, was increased in the presence of muscle juice with citrate, $\alpha$-ketoglutarate, glutamate, or the $C_4$ acids. (Cocarboxylase addition caused a further increase in respiration.) Malonate inhibited the respiration in the presence or absence of insulin, and respiration was restored by fumarate,—citrate and glutamate were much less effective. Injection of malonate into rabbits was found to counteract somewhat the fall in blood sugar caused by insulin injection and to prevent convulsions. The insulin effect does seem to be connected with

[18] In the fourth week some of their birds recovered in health and the insulin effect almost disappeared. The pancreatectomy was not claimed to be complete; it is perhaps possible that regeneration of islet tissue occurred with the birds which recovered.

the activity of the citric acid cycle or the Szent-Györgyi scheme, but the mechanism of the effect is quite uncertain. It may be connected with the proper provision of oxidizable substrate.

d. *Remarks on the citric acid cycle, Szent-Györgyi's theory, and the cycle in kidney cortex slices.* In the light of Krebs' latest papers, it seems very probable that the citric acid cycle theory does describe the main method of carbohydrate oxidation in pigeon muscle and very likely in other tissues. Nevertheless the contradictory results of various authors indicate that a number of points remain to be settled. Several authors have found only small effects of citrate and Thomas finds the action of citrate is delayed compared with that of fumarate. It is possible that the condition of the birds used, with regard to nutrition and previous activity, may affect the *in vitro* behavior of pigeon muscle. Details of technique may also have varied in unnoticed but critical ways. Coenzymes, for instance, may be destroyed more readily under some circumstances than under others. Krebs pointed out that the rates of $O_2$ uptake found by some workers were often lower than his own. With liver, Elliott and Elliott were able to prove catalytic effects with the substances in question by studies of R.Q. and acid disappearance. But the respiration of the liver suspension fell off too soon to be able to show an oxygen uptake increase greater than the equivalent of the added substances. Liver suspension lost its activity rapidly, but intact excised liver could be kept for hours and still showed full activity when brought into suspension and tested at once.

It is not to be expected that the addition of any member of the cycle will always have the same immediate effect as any other. The rate of a complete "tour" of the cycle will depend upon the rate of the slowest individual reaction. The rate of citrate oxidation may not be the limiting factor while the rate of malate oxidation or citrate synthesis may be. In the latter case time may be required to build up the malate or oxaloacetate concentration sufficiently to give the maximum rate.

Citrate, in moderately high concentrations, often shows an inhibitory action. This may be due to inhibition of the citrate oxidizing system itself (see e.g., Bernheim, 24) or to a competitive inhibition of other oxidations resembling the effect of malonate. Inhibition by citrate may be sufficient to mask the catalytic effect until excess citrate has been removed. Further, it should be remembered that citrate (*iso*citrate) oxidation requires coenzyme II while malate oxidation requires coenzyme I. If a deficiency of coenzyme II is produced in the preparation of the tissue, time may be required to rebuild it from coenzyme I by

*phosphorylation reactions* (1). The observation of Banga *et al.* (14) that oxidation of pyruvate by dialyzed brain suspensions was less complete, i.e., less oxygen was taken up per molecule of pyruvate disappearing, when no adenylpyrophosphate was added to the mixture, suggests the possibility that a failure to produce coenzyme II by phosphorylation caused the cycle to be checked at the citrate stage.

Breusch and Stare and Baumann found that citrate was less effective than fumarate in increasing respiration in the presence of malonate. The tendency of added citrate to cause inhibition, added to the action of malonate, may be sufficient to depress the catalytic action of citrate. The inhibition by malonate is overcome when sufficient succinate accumulates to compete successfully with the malonate for the succinic dehydrogenase. But even before the succinate stage is reached, four oxidation steps, each involving an atom of oxygen, can proceed with fumarate, without hindrance by malonate, so that oxygen uptake can *be appreciable from the start. On the other hand, only two oxidation* steps after citrate can occur until a high enough succinate concentration is built up for the cycle to proceed further. $\alpha$-ketoglutarate was more effective than citrate possibly because it is more rapidly broken down to succinate.

Krebs (79) was satisfied that the malate $\rightleftharpoons$ oxaloacetate part of Szent-Györgyi's theory represented the main mediation of H transfer in the oxidation reactions of the citrate cycle, but he was not sure of the further mediation of succinate-fumarate. There is no doubt that this latter mediation can occur, and Krebs has shown it to be essential in the oxidation of various metabolites by *B. coli.* But, as was pointed out on page 279, the discovery of diaphorase makes it unnecessary to assume the mediation of succinate $\rightleftharpoons$ fumarate in all cases. The experiments of Banga (p. 275) in which the addition of yellow enzyme to the system caused increased respiration, might be explained as partly due to the yellow enzyme mediating direct malate oxidation through coenzyme. (The old yellow enzyme, flavin-nucleotide-protein, is related in structure and properties to diaphorase, flavin-adenine-dinucleotide-protein.)

It seems probable that distinct modifications of Krebs' cycle must occur in some tissues. The processes which take place in kidney cortex slices, described earlier, seem very similar to the citric cycle of muscle, and oxidations involved in the kidney cycle may also be mediated by oxaloacetate $\rightleftharpoons$ malate. But an essential difference is *that the members* of the cycle get rapidly used up in kidney cortex slices. The oxaloacetate formed in the cycle may be resynthesized to citrate, but this

must occur without the entry into the cycle of extra pyruvate molecules from outside the cycle. Whatever the process is, it probably occurs to some extent in various minced tissues, when an excess of the catalytic substances is added. Krebs and Eggleston (81, 82) found that added lactate or pyruvate was used up by minced muscle but had little effect on the rate of respiration during the first 30 to 60 minutes, presumably because the enzymes were kept nearly saturated with pyruvate formed from carbohydrate in the tissue. With liver suspension, Elliott and Elliott (46) found that addition of lactate and pyruvate increased the respiration immediately, as much as did citrate and the $C_4$ substances. This may mean that, in liver suspension at least, pyruvate can be rapidly transformed into the catalytic substances by a route not involving synthesis with a pre-formed sufficiency of oxaloacetate. The same holds for kidney slices, since, when pyruvate was added, more succinate accumulated in the presence of malonate than in its absence (47), yet the inhibition of succinate oxidation would cause little oxaloacetate to be available.

The citrate cycle, or a modification of it, is almost certainly not the the only process by which pyruvate is oxidized. A dismutation reaction of pyruvate, yielding acetic acid, is mentioned in the next section, acetic acid appears to be formed from pyruvate by various tissues (85, 100) and by bacteria, and it is well known that acetoacetate can be formed from pyruvate by liver (52, 3, 85, 86; see also p. 279).

As mentioned in a previous section, Elliott and co-workers found that, while the $C_4$ compounds were rapidly oxidized away by kidney cortex slices, with other sliced tissues tried, added fumarate had little effect on the respiration. Such negative results with sliced tissues do not disprove a catalytic rôle for the $C_4$ compounds. In sliced tissues the cells can probably retain their original supply of the $C_4$ substances. Added fumarate or malate may be oxidized to oxaloacetate to a small extent, but any accumulated oxaloacetate would inhibit further malate oxidation (see Banga, 11, 16; Green, 59), and the rate of respiration would continue to be limited by the rate at which oxaloacetate is removed by reduction or by synthesis to citrate. (See discussion by Greig *et al.*, 64.) It is possible that the respiration of slices and other tissue preparations may depend on this type of catalysis and yet not always respond to addition of the catalytic substances or malonate since, as Szent-Györgyi emphasized, the $C_4$ substances may be firmly bound to enzyme proteins in sufficient amount to carry all the respiration and not be rapidly displaced by malonate.

Finally, it should be borne in mind that the transamination reactions,

discussed later, may have important effects on the catalysis of tissue respiration by removing or producing $\alpha$-ketoglutaric and oxaloacetic acids.

KETO-ACIDS—HYDROXY-ACIDS. The position of pyruvic acid as an intermediate in carbohydrate breakdown is now well known. Pyruvic and other $\alpha$-keto-acids are also intermediates in protein metabolism through the oxidative deamination of amino-acids.

Tissue slices under aerobic conditions cause the oxidation of lactate primarily to pyruvate,[19] but added pyruvate becomes partly reduced to lactate (Elliott *et al.*, 49). Krebs and Johnson (85) and Elliott *et al.* pointed out that the lactate-pyruvate system may act as a mediator of hydrogen transport between systems which reduce pyruvate and the system which reoxidizes lactate.

Under anaerobic conditions, Krebs and Johnson found that slices of animal tissues, especially testis, metabolize pyruvic acid by means of a dismutation, yielding lactic and acetic acids and $CO_2$ according to the following equation:

$$2CH_3CO \cdot COOH + H_2O = CH_3CHOH \cdot COOH + CH_3COOH + CO_2$$

Elliott *et al.* simultaneously, but by different methods, also observed the dismutation with testis but found only slight anaerobic $CO_2$ evolution from pyruvate with other tissues.[20] Though all the tissues produced lactate from added pyruvate to some extent, this was probably due to reduction of pyruvate by other systems. The rate of anaerobic $CO_2$ evolution from pyruvate was not rapid enough, except with testis, to account for the whole rapid aerobic pyruvate removal as due to dismutation. Nevertheless it appears that various tissues can remove pyruvate by dismutation with itself.

Krebs (77) found that *Gonococcus*, *Staph. aureus* and *albus*, and *Strept. faecalis* (but not a number of other bacteria) brought about a dismutation of pyruvate yielding lactate, acetate, and $CO_2$. He found that anaerobic $CO_2$ evolution was as great or greater than the aerobic and he concluded that the whole pyruvate metabolism occurred through the dismutation, and that the oxygen uptake observed aerobically was

[19] Greig *et al.* (64) have indicated that ox retina oxidizes added lactic acid to give pyruvic acid and some acid other than pyruvic or acetic. The unknown acid may have been formed in other tissues but differing relative rates of reaction may have prevented it from being apparent.

[20] The reason for the greater effects found by Krebs and Johnson is not clear. These authors used a bicarbonate-containing "saline" at 39–40° while Elliott *et al.* used the bicarbonate-containing medium of Krebs and Henseleit (83) at 37–38°.

due to reoxidation of the lactate produced. Barron and Lyman (21), however, showed that aerobically direct oxidation of pyruvate also occurred.

Probably the essential reaction in both dismutation and oxidation (other than oxidative syntheses giving citrate or acetoacetate) is an oxidative decarboxylation of pyruvate to acetate and $CO_2$; in dismutation other molecules of pyruvate act as primary hydrogen acceptor, in aerobic conditions oxygen is the ultimate hydrogen acceptor (see e.g., Lipmann, 99). Since lactate can be reoxidized to pyruvate, the pyruvate-lactate system may be regarded as a carrier for the oxidation of pyruvate itself. The system may be represented as follows:

$$\begin{array}{lccccl}
 & & CH_3 & & CH_3 & \\
 & & | & & | & \\
\text{pyruvate} & \xrightarrow{2H} & CO & \rightleftharpoons & CHOH & \xrightarrow{2H} \text{other carrier systems} + O_2 \\
\downarrow & & | & & | & \\
 & & COOH & & COOH & \\
 & & \text{pyruvate} & & \text{lactate} & \\
\text{acetate} & & \multicolumn{3}{c}{\text{Activated by the lactic}} & \\
+ & & \multicolumn{3}{c}{\text{dehydrogenase system}} & \\
CO_2 & & & & &
\end{array}$$

Besides pyruvate, other keto-acids may act as carriers. In various sliced tissues, Krebs and Johnson (85) showed the occurrence of dismutative oxidations of pyruvate by acetoacetate and oxaloacetate, these substances being reduced to $\beta$-hydroxybutyrate and malate respectively. Further, $\alpha$-ketoglutarate could be oxidized by acetoacetate giving succinate, $CO_2$, and $\beta$-hydroxybutyrate. Weil-Malherbe (150) showed the dismutation of $\alpha$-ketoglutarate with itself in brain tissue, succinate, $CO_2$, and $\alpha$-hydroxyglutarate being formed; evidence for the same reaction in kidney cortex was obtained by Krebs and Cohen (80).

All the reduction products of these dismutations,—lactate, $\beta$-hydroxybutyrate, $\alpha$-hydroxyglutarate, and malate,—are known to be reoxidizable by tissues under aerobic conditions. The dismutations were shown to occur anaerobically. It is probable that they also occur in the presence of oxygen and that keto $\rightleftharpoons$ hydroxy-acid mediates aerobic oxidation of keto-acids. However, with pyruvate, and almost certainly with the other keto-acids, oxidation without this type of mediation also occurs in tissues.

It is probable that the carrier function of keto-hydroxy-acids is not limited to oxidations of keto-acids themselves. With isolated enzyme systems, dismutations have been demonstrated (Euler *et al.*, 53; Dewan and Green, 42; Green *et al.*, 60, Dewan, 41) whereby pyruvate and oxalo-

acetate accept hydrogen from triosephosphate, $\alpha$-glycerophosphate, $\beta$-hydroxybutyrate and glutamate. The dismutation between pyruvate and triosephosphate is, of course, an essential step in the process of glycolytic lactic acid formation; the similar dismutation between acetaldehyde and triosephosphate which occurs in alcoholic fermentation should also be mentioned. The oxaloacetate-malate reaction is a part of Szent-Györgyi's scheme and, as discussed in previous sections, it is probably involved in a variety of oxidations; other keto-acids may play a similar rôle. An oxidation of citrulline by $\alpha$-keto-acids, giving glutamic acid and urea, has been suggested as one of the mechanisms of urea formation in liver slices (Bach, 10).

Szent-Györgyi (140) proposed a hypothesis to explain why, in many tissues, lactic acid accumulates under anaerobic conditions but not under aerobic conditions (Pasteur reaction). Laki (90) had found that, when oxaloacetate was added to muscle extract, it was reduced to malate while pyruvate was formed by the oxidation of a donator, presumably "triose" (triosephosphate). The pyruvate was later oxidized away. It was suggested that, under both anaerobic and aerobic conditions, tissues cause "triose" to be dehydrogenated, losing two H atoms, and rearranged to give pyruvate. Anaerobically, pyruvate itself accepts the two H atoms giving lactate. Aerobically oxaloacetate takes up the hydrogen giving malate, but this is reoxidized by the fumarate-succinate-WKS-$O_2$ system, so that a supply of oxaloacetate is maintained. The pyruvate, aerobically, is oxidized away or re-synthesized to carbohydrate. Evidence was provided (Das, 40; Laki, 92) that oxaloacetate, if present, would accept the hydrogen more readily than would pyruvate. This scheme has been strongly supported by Parnas and Szankowski (115, see also 107).

Glutamic and aspartic acids. Among the products of protein metabolism, the two dibasic amino acids, glutamic and aspartic, have lately been shown to play a special part in oxidative metabolism.

Braunstein and co-workers (33, 31) found that a type of reversible oxidation-reduction process, in which a transfer of amino-groups as well as of hydrogen atoms occurs, takes place in most animal tissues, in plants, and in micro-organisms. This type of reaction has been called "transamination" and the following equations represent examples:

$$\underset{\text{glutamic acid}}{COOH\cdot CH_2\cdot CH_2\cdot CHNH_2\cdot COOH} + \underset{\text{pyruvic acid}}{CH_3\cdot CO\cdot COOH} \rightleftharpoons$$
$$\underset{\alpha\text{-ketoglutaric acid}}{COOH\cdot CH_2\cdot CH_2\cdot CO\cdot COOH} + \underset{\text{alanine}}{CH_3\cdot CHNH_2\cdot COOH}$$

$$\underset{\text{aspartic acid}}{COOH\cdot CH_2\cdot CHNH_2\cdot COOH} + \underset{\text{pyruvic acid}}{CH_3\cdot CO\cdot COOH} \rightleftharpoons \underset{\text{oxaloacetic acid}}{COOH\cdot CH_2\cdot CO\cdot COOH} + \underset{\text{alanine}}{CH_3\cdot CHNH_2\cdot COOH}$$

For transamination to occur, one of the reactants, the "primary substrate," must be a dicarboxylic $\alpha$-amino-acid or $\alpha$-keto-acid or a compound having similar electrostatic qualities, such as phosphoserine, cysteic, or homocysteic acid. The other reactant may be an $\alpha$-amino- or $\alpha$-keto-acid other than glycine; glycine, $\beta$- and $\gamma$-amino- or keto-acids, simple amines, aldehydes, ketones, and peptides, do not react. Transamination occurs selectively, and possibly exclusively, with the natural, *l*-series, amino-acids. Since the reactions are reversible, small amounts of amino- or keto-dicarboxylic acids can act as mediators in amino-group transfers between pairs of mono-carboxylic amino- and keto-acids. Transaminations are catalyzed by enzymes called "aminopherases". There are apparently two of these, since preparations have been obtained (Kritzmann, 88) which act only with glutamic acid or only with aspartic acid as primary substrate; both enzymes seem to require a coenzyme.

Euler, Adler *et al.* (54), in their studies on glutamic acid dehydrogenase, have shown that glutamic acid can be produced from $\alpha$-keto-glutaric acid and $NH_3$ with dihydro-coenzyme (either coenzyme I or II with the enzyme from animal tissue) under the influence of the apo-dehydrogenase. The $\alpha$-keto-glutaric acid and $NH_3$ reversibly and spontaneously give the imino-acid which is reduced by the dihydro-coenzyme. The glutamic acid formed, with aminopherase, can then transfer the amino-group to various $\alpha$-keto-acids. Thus by the coöperation of glutamic dehydrogenase, aminopherase, a coenzyme-determined dehydrogenase, coenzyme, and a trace of glutamic or $\alpha$-keto-glutaric acid, a synthesis of an amino-acid may be coupled with the oxidation of another substance. The following equations summarize the reactions involved:

$$\text{donator} + \text{coenzyme} \underset{\textit{donator apodehydrogenase}}{=} \text{oxidized donator} + \text{coenzyme } H_2$$

$$\alpha\text{-ketoglutarate} + NH_3 = \alpha\text{-iminoglutarate} + H_2O$$

$$\alpha\text{-imino-glutarate} + \text{coenzyme } H_2 \underset{\textit{glutamic apodehydrogenase}}{=} \text{glutamate} + \text{coenzyme}$$

$$\text{glutamate} + \alpha\text{-keto-acid} \underset{\textit{aminopherase}}{=} \alpha\text{-keto-glutarate} + \text{amino-acid}$$

*Net effect*

$$\text{Donator} + \alpha\text{-keto-acid} + NH_3 = \text{oxidized donator} + \text{amino-acid} + H_2O$$

A similar series of reactions mediated by aspartic instead of glutamic acid is possible, particularly in plants; aspartic acid may also be produced from $NH_3$ and fumarate under the influence of aspartase (see 31).

Du Vigneaud and Irish (146), following earlier suggestions of Knoop, have indicated that amino-acids may be synthesized in the body from $\alpha$-keto-acids and $NH_3$ by an oxidation-reduction reaction with pyruvic acid whereby $CO_2$ is liberated and the acetyl-amino-acid is formed. The latter is then hydrolyzed to acetic acid and the amino-acid.

Braunstein suggests that glutamic (or aspartic) acid with its aminopherase and dehydrogenase may be concerned in the oxidation of the natural, *l*-series, amino-acids. Since the work of Bernheim and Bernheim (25) and Krebs (75), the presence in tissues of an enzyme which causes the oxidative deamination of *d*-amino-acids has become well known; this enzyme belongs to the group of flavin-adenine-dinucleotide-protein complexes. But the system responsible for the oxidation of the natural amino-acids appears to be much more complex and labile; no simple *l*-amino-acid oxidizing enzyme, other than glutamic dehydrogenase, can be separated from animal tissues. Braunstein suggests that the *l*-amino-acids undergo transamination with $\alpha$-keto-glutarate yielding the $\alpha$-keto-acids and glutamic acid; the latter is then reoxidized to $\alpha$-keto-glutarate by glutamic dehydrogenase, with the liberation of $NH_3$. The reactions involved may be represented as follows:

$$l\text{-amino-acid} + \alpha\text{-keto-glutarate} \underset{\textit{aminopherase}}{=} \alpha\text{-keto-acid} + \text{glutamate}$$

$$\text{glutamate} + \text{coenzyme} \underset{\textit{glutamic apodehydrogenase}}{=} \text{imino-glutarate} + \text{coenzyme}\cdot H_2$$

$$\text{coenzyme}\cdot H_2 + O \underset{\textit{diaphorase and the cytochrome system}\ \text{(probably)}}{=} \text{coenzyme} + H_2O$$

$$\text{imino-glutarate} + H_2O = \alpha\text{-keto-glutarate} + NH_3$$

*Net effect*

$$l\text{-amino-acid} + O = \alpha\text{-keto-acid} + NH_3$$

Braunstein (32) has actually set up an artificial mixture of enzymes, coenzymes, and $\alpha$-keto-glutarate, which will bring about the oxidation of certain *l*-amino-acids.

Virtanen and Laine (147) have indicated that in root nodules of leguminous plants, hydroxylamine, produced in the fixation of nitrogen, may combine with oxaloacetic acid to give oximinosuccinic acid which is reduced to aspartic acid. The aspartic acid may then transfer the amino-group to the other $\alpha$-keto-acids giving rise to amino-acids.

Braunstein (31) suggests a number of other ways in which transaminations may affect respiration and metabolism.

The extent to which such processes as those described above can take place in various tissues depends on how many of the amino- or keto-acids can take part in transaminations. Cohen (36) found that, in pigeon breast muscle, out of 21 amino-acids tried, only aspartic acid and alanine react rapidly with $\alpha$-keto-glutarate; valine and $\alpha$-amino-butyric acid reacted slightly. Of a series of $\alpha$-keto-acids, only oxalo-acetic and pyruvic acids reacted rapidly with glutamic acid. However, in liver and kidney cortex Cohen (37) has found much wider reactivity. In liver appreciable reaction occurred between $\alpha$-keto-glutaric acid and aspartic acid, alanine, arginine, iso-leucine, leucine, valine, phenyl-alanine, and histidine. Kidney cortex was active with all these, except histidine, and also with cysteine, methionine and lysine. No reaction occurred with glycine, proline, tryptophane, or any *d*-amino-acid.

Krebs and Cohen (80) found with kidney cortex slices or minced heart muscle, but not appreciably with other tissues, that added $\alpha$-keto-glutarate and $NH_3$ undergo the following reaction:

$$2\alpha\text{-keto-glutarate} + NH_3 = \text{glutamate} + \text{succinate} + CO_2$$

They considered that the reaction was essentially a dismutation between $\alpha$-iminoglutarate, formed from $\alpha$-keto-glutarate and $NH_3$, and unchanged $\alpha$-keto-glutarate, as follows:

$$\alpha\text{-iminoglutarate} + \alpha\text{-keto-glutarate} + H_2O = \text{glutamate} + \text{succinate} + CO_2$$

Since glutamate can be reoxidized to $\alpha$-iminoglutarate, the system $\alpha$-iminoglutarate $\rightleftharpoons$ glutamate may act as a hydrogen transporting system. It had been observed previously (Krebs, 74; Edson, 44) that addition of $NH_4Cl$ catalytically increased the respiration of kidney cortex in the presence of $\alpha$-keto-glutarate or substances, such as lactate, pyruvate, or glucose, which might give rise to $\alpha$-keto-glutarate.

OTHER SYSTEMS. *Formate-bicarbonate.* Krebs (76; see also 109; 39) found that *B. coli.*, in bicarbonate-containing medium under anaerobic conditions, brings about the reaction:

$$\underset{\text{pyruvic acid}}{CH_3\cdot CO\cdot COOH} + H_2O = \underset{\text{acetic acid}}{CH_3\cdot COOH} + \underset{\text{formic acid}}{HCOOH}$$

He considered that this reaction takes place in the following manner:

$$CH_3\cdot CO\cdot COOH + O \mid H_2 + CO_2 = CH_3\cdot COOH + CO_2 \mid + HCOOH$$

The $CO_2$ or bicarbonate of the medium accepts hydrogen, giving formate. Since, under aerobic conditions, *B. coli* causes the oxidation of

formate to $CO_2$ (bicarbonate), the formate-bicarbonate system may act as a carrier in the oxidation of pyruvate and other substances in certain organisms. There is no evidence for such a possibility in animal tissues.

*Catechol-o-quinone.* It is probable that the polyphenol oxidase-polyphenol system performs in certain plant tissues a similar function to that of the cytochrome oxidase-cytochrome system. Long ago, Palladin (114) suggested that the oxidation products formed by polyphenol oxidase (catechol oxidase) action oxidize cell substances. Kubowitz (89) observed that o-quinone, formed by the action of catechol oxidase on catechol, can oxidize the dihydro-coenzymes I and II, and this shows that catechol-o-quinone, with catechol oxidase, can act as a hydrogen transporting system for all the coenzyme-determined dehydrogenase systems. Peroxidase and $H_2O_2$ also oxidize phenols so that the catechol-o-quinone system can mediate oxidations by $H_2O_2$ when peroxidase is present.

Catechol and derivatives of it are widely distributed among plant tissues. Boswell and Whiting (29) obtained evidence that as much as 66 per cent of the respiration of potato slices was catalyzed by the catechol system.

*Ascorbic acid.* Ascorbic acid oxidation can be catalyzed by ascorbic oxidase (139, 143), by o-quinones (138, 69, 72), by cytochrome-cytochrome oxidase (138, 19), and by Cu and other compounds (138, 133, 20). Tissues reduce oxidized ascorbic acid (dehydro-ascorbic acid) rapidly and ascorbic acid may serve some function as mediator of oxidations. However, no dehydrogenase systems are known to reduce dehydro-ascorbic acid, though glutathione and protein-SH groups can do so (138, 28, 121, 68). Szent-Györgyi (139) indicated that the ascorbic acid system plays an important part in mediating the respiration of cabbage leaves.

*Dihydroxymaleic acid.* Banga and Szent-Györgyi (18) discovered that plant tissues contain an enzyme which causes the vigorous oxidation of the $C_4$-dicarboxylic acid, dihydroxymaleic acid. The oxidation of this substance was also catalyzed by inorganic iron or copper. The substance could also be oxidized indirectly by oxygen, or by $H_2O_2$ with peroxidase, through certain phenol-quinones (Robeznieks, 120; Philippot, 116). The oxidation product, diketosuccinic acid, was believed to be re-reduced by tissue systems and so may act as a mediator of respiration (Banga and Philippot, 15, Philippot 116).

*Adrenochrome.* Adrenaline can be oxidized by the cytochrome-

cytochrome oxidase system, or by catechol oxidase, to adrenochrome,[21] N-methyl-2,3-dihydro-3-hydroxyindole-5,6-quinone (Green and Richter, 61). Green and Richter found that this red pigment could accept hydrogen from reduced coenzyme I and the leuco-adrenochrome formed could then be reoxidized either by the cytochrome-cytochrome oxidase system or by another, cyanide insensitive, system which was not further studied. In this way adrenochrome could mediate oxygen uptake with a number of coenzyme-dehydrogenase systems. From the activity *in vitro* of low concentrations of adrenochrome, Green and Richter concluded that the carrier function of this hormone derivative might be of physiological significance.

*Stickland reaction.* Stickland (132, see also 151, 56) discovered that the anaerobic organism, *Clostridium sporogenes,* can produce energy for growth by the oxidative deamination of one group of amino-acids while certain other amino-acids act as hydrogen acceptors.

## CONCLUSION

Until quite recently studies on tissue respiration and the mechanisms which promote biological oxidation-reduction reactions, have been carried on rather independently of other fields of chemical physiology. The work described above has shown that the fields of intermediary metabolism and respiratory catalysis cannot be separated, since substances which are known to be intermediate products of the breakdown of foodstuffs are also parts of the catalytic mechanisms for the energy-yielding oxidations of such foodstuffs.

The synthesis of amino-acids from keto-acids and $NH_3$, and the breakdown of the *l*-amino-acids may involve the mediation of the dibasic amino-acid-keto-acid system and coupled oxidation or reduction of other substances. Work of the last few years has shown that various oxidation-reductions are coupled with phosphorylation of glucose and other compounds; as a result, studies of tissue oxidations have become connected with the study of glucose and glycogen synthesis and breakdown, and various other processes which involve phosphoric acid esters. The discovery of the coupling of phosphorylation with oxidation-reduction has provided an understandable mechanism whereby the energy of oxidations is made available for certain syntheses.

Tissue respiration studies are now intimately connected with the

[21] Adrenaline can also be oxidized by animal tissue preparations to give, primarily, 3,4-dihydroxy-phenyl-glycollic aldehyde, through the action of adrenaline or amine oxidase (Blaschko et al., 27).

science of nutrition, since several of the B group of vitamins are known to be parts of the prosthetic groups of oxidation enzymes. The field of endocrinology is impinged upon since a definite effect of insulin on respiration is now known, a carrier function for adrenochrome is suspected, and an oxidative enzyme (amine oxidase) causes the inactivation of adrenaline. Proteolytic enzyme chemistry is connected to oxidation-reduction chemistry through the action of glutathione, ascorbic acid, and other reversibly oxidizable systems, on the —SH groups which are necessary for the activity of some proteolytic enzymes.

More than ever biochemists need to have the "biological outlook," the recognition of the complex interlocking of dynamic systems in biological materials. One aspect of this interlocking has been outlined in the preceding pages.

*Appendix.* Since this review went to press several further papers concerning the citric acid cycle have appeared.

Smyth (156), in Kreb's laboratory, has shown the rapid oxidative utilization of pyruvate by minced sheep heart. He applied the same type of experimentation as was used by Krebs and Eggleston (82) with pigeon breast muscle (p. 287) and obtained very similar results. He concluded, in agreement with Hallman and Simola (66), that the citric acid cycle plays a part in the metabolism of heart tissue. He noted that citrate, especially in raised concentrations, has a smaller effect on the respiration than the other substances concerned in the cycle and occasionally even increased the inhibition with malonate.

Evans (152), also in Kreb's laboratory, has shown that in minced pigeon liver, oxaloacetate and pyruvate react to form citrate and $\alpha$-ketoglutarate and added citrate is oxidized, indicating that the citric acid cycle can occur in liver. But in the presence of added pyruvate, the oxygen uptake and the very rapid pyruvate utilization are not inhibited by malonate, and the addition of fumarate with or without malonate has no effect. With high concentrations (0.02–0.04 M) of pyruvate, an accumulation of $\alpha$-ketoglutarate occurs, and, in the presence of malonate, acetoacetate and succinate also accumulate. The formation of acetoacetate from pyruvate by liver mince has been shown previously (see pp. 279 and 293). The formation of $\alpha$-ketoglutarate in liver evidently does not require the addition of $C_4$ dicarboxylic acid (see also p. 293). But $\alpha$-ketoglutarate and citrate are formed from oxaloacetate plus pyruvate anaerobically much more rapidly than from pyruvate alone. It is therefore probable that, at low concentrations, pyruvate is metabolized largely by way of the citric acid cycle.

Krebs *et al.* (154) found that guinea-pig kidney, as well as pigeon breast muscle and sheep heart, with added oxaloacetate in anaerobic conditions, gives $CO_2$, fumarate, malate, $\alpha$-ketoglutarate, citrate and succinate in the amounts to be expected from the citric acid cycle theory with oxaloacetate acting as hydrogen acceptor. The oxaloacetate also provides, by its own decomposition some of the pyruvate necessary for the citrate synthesis.

In sheep brain and testis, Krebs *et al.* found citrate and $\alpha$-ketoglutarate formation from oxaloacetate suggesting the occurrence of the cycle or some of its

component reactions also in these tissues. But no conclusion could be drawn as to the quantitative importance of these reactions in brain and testis in view of the results obtained by Banga *et al.* (14) with brain (p. 286) and by Krebs and Johnson (85) and Elliott *et al.* (49) with testis (p. 294).

Using bicarbonate containing labelled, radioactive, carbon, Evans and Slotin (153) have shown that carbon dioxide in the medium participates in the synthesis of $\alpha$-ketoglutarate from pyruvate in pigeon liver. Presumably the carbon dioxide combines with pyruvate giving oxaloacetate which then combines with more pyruvate to give $\alpha$-ketoglutarate via citrate. This observation would explain Evans' observation, mentioned above, that the formation of $\alpha$-ketoglutarate by liver does not require the addition of $C_4$ dicarboxylic acid. Krebs (155) also mentions that he has evidence for the carboxylation of pyruvate in pigeon liver. He states that experiments on vitamin $B_1$ deficient pigeons suggest that the reaction between carbon dioxide and pyruvate is the stage at which the vitamin enters the metabolism of pyruvate in animal tissues and that the vitamin is not concerned with the direct oxidation of pyruvate.

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# THE EFFECT OF ANOXIA ON THE ALIMENTARY TRACT

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The effect of anoxia on respiration, on the blood and circulation, and on the central nervous system has interested physiologists for over a half-century. Studies on the effect of anoxia on the alimentary tract, however, relatively have been neglected. One reason for this may be that the effects of anoxia on the alimentary tract are not manifested as strikingly as on other organs or systems of the body; moreover, observations on the gastrointestinal tract are more difficult to make since cumbersome apparatus is often needed which precludes making observations at high altitudes.

It is known that the effect of anoxia depends upon the length of sojourn at high altitudes and on the degree of the severity of the anoxia. In this review the degree of anoxia will be expressed as partial pressures of oxygen in millimeters of mercury, and in order to help orient the reader the approximate altitude in feet will also be given.

Since the oxygen percentage composition of the atmosphere remains practically unaltered, at least to a height of 72,000 feet (39), it is confusing to express altitude in percentage of oxygen. The latter expression may be used, however, if a known percentage of oxygen is delivered at a given concentration by some mechanical device. The degree of anoxia may also properly be expressed by stating the actual barometric pressure.

In this review, four types of anoxia will be recognized: 1, the anoxia type; 2, the anemic type; 3, the stagnant type; 4, the histotoxic type.

*Effect on motility.* As far as the author is aware, no work has been reported in the literature on the effect of anoxia on swallowing movements. The mechanism is a complicated one and it is doubtful whether the normal act of swallowing is entirely understood. No work, furthermore, has been reported on the effect of anoxia on the esophagus or on the cardia. Most of the work dealing with the effect of anoxia on the movements of the gastro-intestinal tract has been done on the stomach; some work also has been reported on the pyloric sphincter.

Effect of anoxic anoxia on gastric motility. *Hunger contractions.* In 1928 Van Liere and Weaver (unpublished work) and later Van Liere and Crisler (42) using the balloon method, showed that anoxia decreased the amplitude of hunger contractions in the normal trained dog. At a partial pressure of oxygen of 80 mm. Hg (18,000 ft.) or even at a somewhat higher partial pressure of oxygen there was a distinct loss of gastric tone and a diminution in the height of the hunger contractions; they still persisted faintly, however, at a partial pressure of oxygen of 40 mm. of Hg.

Hellebrandt, Brogdon and Hoopes (20) in 1935 studied the effect of acute anoxia on hunger contractions in man. Their subjects were allowed to breathe 8 per cent oxygen or less from 5 to 14 minutes out of a closed system; the carbon dioxide was removed by soda lime. These authors reported that although motility seemed more susceptible to anoxia than secretion, the degree of change was not marked. They concluded that the pre-coma type of anoxia had but little effect on hunger contractions. Krugly (21) in 1938, working with dogs, reported that hunger contractions were inhibited at a barometric pressure from 320 to 357 mm. Hg.

*Motility of the stomach containing food.* Using both the balloon method and the gastrograph, Crisler, Van Liere and Booher (8) working with barbitalized dogs, found that a partial pressure of oxygen of 80 mm. Hg (18,000 ft.) or somewhat less, caused inhibition of gastric digestive motility. There was a decreased amplitude of contraction and a fall of gastric tone. At a partial pressure of oxygen of 50 mm. Hg the digestive contractions were greatly diminished in height but were not abolished. No retching or vomiting movements were reported. The authors felt that the most plausible mechanism for the early inhibition of digestive contractions produced by anoxia was a sensitization of the sympathetics by the rise in pH accompanying the initial hyperpnea and hyperventilation in anoxia.

Hellebrandt, Brogdon and Hoopes (20) worked on the effect of acute anoxia on digestive contractions in the human using the same technique and same percentages of oxygen as was used in their work on hunger contractions. They reported that anoxia moderately depressed the motor activity of the stomach, but concluded that the pre-coma type of anoxia had relatively little inhibitory effect upon digestive motility. Since these authors used an entirely different technique from that reported by Crisler, Van Liere and Booher, the results obtained by the two different groups of workers are not incompatible.

*Effect on motility of gizzards of birds.* Nolf in 1925 published several papers (25, 26, 27) on the influence of hypercapnia and anoxia on the motility of the gizzards of birds. He found that by stimulating the extrinsic nerves of the gizzard for a few seconds, contractions were set up which continued for about 15 minutes. If the animals were subjected to an 8 or 10 per cent oxygen mixture in hydrogen or to air or hydrogen mixed with 5 per cent carbon dioxide, the contractions of the gizzard would be greatly depressed or even inhibited. As soon as the birds were removed from the influence of anoxia or the carbon dioxide mixture, the contractions of the gizzard would be resumed. He further showed that the adrenals played a part in inhibiting the contractions produced by anoxia; while extirpation of these organs did not abolish the effects of anoxia, the response was slower after their removal.

*Effect on gastric emptying time.* In 1933 Van Liere, Crisler and Robinson (43) reported that dogs subjected to anoxia showed a delay in the emptying time of the stomach. At a partial pressure of oxygen of 117 mm. of Hg (8,000 ft.) three of four dogs showed a delay in gastric emptying as determined by the fluoroscope. The authers considered this degree of anoxia a threshold for the normal dog. The more severe the degree of anoxia the greater was the prolongation of tho empyting time of the stomach. At a partial pressure of oxygen of 73 mm. of Hg (20,000 ft.) the 2 dogs subjected to this degree of anoxia still had food in their stomach at the end of 24 hours. Later work (unpublished) on 23 additional dogs fully corroborated the work reported previously which was based on 6 animals. Krugly (21) in 1938 also reported that gastric emptying in dogs was prolonged by oxygen want.

In 1936 the author assisted by Lough and Sleeth (47) reported work on the effect of anoxia on the emptying time of the human stomach. Eight young male adults were used as subjects. Anoxia was produced by placing the subjects in a low-pressure chamber (41) and gastric emptying was determined by use of the fluoroscope. The work with human beings correlated well with that found in the dog although the dogs appeared to be somewhat more susceptible to anoxia. At a partial pressure of oxygen of 117 mm. Hg (8,000 ft.) 2 of 3 subjects showed a slight but definite prolongation of gastric emptying. The authors gave this as the probable threshold for the average unacclimatized individual. At a partial pressure of oxygen of 94 mm. Hg (14,000 ft.) all of the 8 subjects showed a delay in gastric emptying time. Marked individual variations were noted; the range varied from 13.2 per cent to 166.9 per cent. Two of the individuals were subjected to a

simulated altitude of 18,000 feet. The authors concluded that with man, as in case of the dog, the more severe the degree of anoxia the greater the delay in gastric emptying.

*Anoxia produced by the administration of nitrous oxide anesthesia.* Sleeth and Van Liere (36), making a comparative study of the effects of various anesthetic agents on gastric emptying in the dog, found that nitrous oxide caused a delay in gastric emptying of 15 per cent. Their interpretation was that the delay was produced principally by anoxia. Since in administering nitrous oxide it is necessary to use a concentration of 95 per cent, the animals were actually getting 5 per cent oxygen during the 15 minutes the anesthesia was given.

*Mechanism of the delay in gastric emptying produced by anoxic anoxia.* Crisler, Van Liere and Wiles (9) working with dogs came to the conclusion that the delay in gastric emptying produced by anoxic anoxia was on a vagospastic pylorospastic basis, that is, the anoxia stimulated the vagus causing some contraction of the pyloric sphincter and this narrowing of the pyloric passage delayed stomach emptying. Anoxia produced this pylorospasm until a critical threshold was reached; beyond this threshold further delay was caused by the oxygen want acting directly on the smooth muscle of the stomach. The authors believed that the same factors were operative in man as in the dog.

Another mechanism must also be considered. If the secretion of epinephrine is capable of being augmented by anoxia, the increased epinephrine secretion could cause a definite inhibition of gastric motility. It has been shown in human beings (48) that ephedrine greatly delays gastric emptying. It is also known that epinephrine may produce a delay in gastric emptying time (37, 59).

*Effect on the pyloric sphincter.* In 1935 Van Liere, Crisler and Wiles (44), working with barbitalized dogs and using a pressure tonometer in the pylorus as described by Thomas (40), studied the effect of anoxic anoxia on the pylorus. A variability in the results was reported which they attributed to the complexity of the control of the pylorus and to the general nature of the stimulus of anoxia. Anoxia caused a rise in the tone in the pyloric sphincter in some animals and a fall in others. The height of the pyloric contractions, however, was generally diminished, especially if severe grades of anoxia were used.

Later studies (53), using a pressure tonometer in the pylorus of unanesthetized dogs with permanent gastric and duodenal fistulae, demonstrated that anoxia produced a rise in the tone of the pyloric sphincter in 14 out of 17 trials. The threshold for the production of this increase of tone in the unanesthetized dog was a partial pressure of

oxygen of 108 mm. Hg (10,000 ft.). The effect of the anoxia on the pyloric contractions was indeterminate. Concomitant tracings were taken of the pyloric antrum; anoxia decreased the tone of the pyloric antrum and effective grades of anoxia abolished the normal rhythmical contractions.

EFFECT OF STAGNANT ANOXIA ON GASTRIC MOTILITY. This type of anoxia may be caused by cardiac failure, impaired venous return, or by shock. While it is known clinically that certain cardiac diseases may produce nausea and vomiting as well as minor gastric disorders, it is difficult to find in the literature any quantitative data or any carefully controlled work on the effect of stagnant anoxia on gastric emptying. In order for the work to be well controlled, it would be necessary to know what the normal gastric emptying time of the patient had been before the stagnant type of anoxia had developed. Other difficulties would present themselves, such as the psychic factor in cardiac disease, the relatively physical inactivity of the patient, and others. Since any of these factors could influence gastric emptying, it would be necessary to evaluate very carefully any results obtained.

EFFECT OF ANEMIC ANOXIA ON GASTRIC MOTILITY. Carlson (6) in 1918 reported work on the effect of acute hemorrhage on hunger contractions. After bleeding two dogs about 30 per cent of their calculated blood volume, he found that the hemorrhage induced temporarily a greater gastric tonus and intensity of hunger contractions than those typical for these dogs before the hemorrhage. This augmented effect disappeared in less than 24 hours. Carlson felt that the tissues deprived of so much nutritive material possibly liberated a hormone which acted on the neuromuscular apparatus of the stomach stimulating hunger contractions. No subsequent proof, however, has been offered for this interpretation.

The effect of acute hemorrhage on gastric motility has been studied in human beings and in dogs by Van Liere, Sleeth and Northup (52). They reported that in the human being, after one-tenth of the calculated blood volume had been withdrawn, the gastric emptying time was prolonged an average of 41 per cent in 4 individuals. In no case was it less than 25 per cent. There was still a delay from 15 to 20 per cent in three of the men 24 hours after the hemorrhage. The stomach had apparently regained its normal motility in all four subjects at the end of 48 hours. Two dogs which too had one-tenth of their calculated blood volume withdrawn, also showed a noticeable delay in gastric emptying thus confirming the results obtained in man.

In 1938 Curtis and Hamilton (10), using the balloon method for ob-

serving gastric motility, reported studies made on patients suffering from pernicious anemia. Prior to the administration of liver therapy, an intense and persistent gastric motility was observed. This persisted during liver therapy and continued even after marked clinical improvement was noticed in the patient. The authors could not account for this hypermotility but suggested that it might have been produced by the associated anacidity or possibly by stimulation of the gastric neuromuscular apparatus by pathological changes in the stomach. These authors, using the same method, also reported continuous motility with frequent contractions of high amplitude in a case of hypoplastic anemia.

No well controlled work could be found in the literature of the effect of a primary anemia or a chronic secondary anemia or the leukemias on the gastric emptying time.

*Carbon monoxide poisoning.* Carbon monoxide exerts its action mainly by uniting with the hemoglobin of the blood and in this way prevents the hemoglobin from transporting the normal amount of oxygen. It therefore produces an anemic type of anoxia. It may, however, also act as a histotoxic agent. Peterson, Smith, and Hale (30), working with rats, reported that chronic carbon monoxide poisoning inhibited gastro-intestinal peristalsis.

Effect of histotoxic anoxia on motility. The cyanides exert the greatest depression on cellular oxidation. They are not, however, widely used in medicine and no reports could be found in the literature of the effect on gastro-enteric motility in the intact animal of cyanides or any other agent unequivocally producing histotoxic anoxia. Alvarez (1), however, has reported that the more highly active portions of the isolated strips of small intestine are inhibited more by cyanides than are less active strips.

There is a difference of opinion as to the degree of histotoxic anoxia produced by the inhalation anesthetics during surgical anesthesia. According to Dr. R. M. Waters (56) general anesthetic agents even when surgical anesthesia is produced, should not, if properly given, produce anoxia. Since all general anesthetic agents, however, produce respiratory depression in some measure, anoxia, unless the anesthesia is carefully given, may be produced by these agents. Anoxic anoxia is particularly difficult to avoid when nitrous oxide is the sole anesthetic agent.

A comparative study of common inhalation anesthetics has been reported on gastric motility in dogs (36). Surgical anesthesia (third plane) was rapidly induced and maintained for 15 minutes. As soon

as the animals regained consciousness they were allowed to eat the test meal. Their normal emptying had been determined previously. Fluoroscopic examination showed that all the anesthetic agents used prolonged gastric emptying time: chloroform by 64 per cent; ether by 40 per cent; nitrous oxide by 15 per cent; ethylene, cyclopropane, and divinyl oxide each by about 7 per cent. The severe anoxia accompanying the nitrous oxide anesthesia probably explains the longer delay with nitrous oxide than with the other gases. Emerson (13), using Macht's technique, found that surgical anesthesia with ether inhibits the motility of the small intestine of rats for the period of its duration. The intestine regained approximately its normal activity by the second hour after anesthesia was terminated.

These results have been interpreted by some authors as being due to sympathetic stimulation but if later work shows that these anesthetic agents exert a histotoxic effect in surgical anesthesia, their action could be explained on this basis.

With the fixed narcotics, effects directly on smooth muscle or on its innervation as a rule outweigh any possible histotoxic anoxia produced.

EFFECT OF ANOXIA ON THE MOVEMENTS OF THE SMALL INTESTINE AND COLON. It can be demonstrated easily that fulminating anoxia may stimulate intestinal peristalsis. If a guinea pig be struck a blow on the head and the abdomen be opened, the small intestines in many instances can be seen to undergo an active peristalsis. This initial stimulation apparently produced in part by the anoxia is, of course, but transitory in nature and is soon followed by a paralysis of the whole intestine.

In 1934 Schnohr (33) studied intestinal peristalsis by inserting an oval cellophane window in the abdominal wall of rabbits. He reported that anoxia or an increase in carbon dioxide concentration in the blood caused violent contraction of the arteries of the intestine and an immediate cessation of all intestinal movement.

The work of Emerson (13) on the effect of ether surgical anesthesia on the motility of the small intestine has been mentioned under the discussion of histotoxic anoxia. Peterson, Smith, and Hale (30), whose work was mentioned when CO poisoning was discussed, used a modification of Macht's technique in studying the effect of CO on gastrointestinal motility in rats. They found that if the blood be saturated from 70 to 80 per cent with CO, gastro-peristalses were inhibited 33 per cent. What they termed, "the egestion time", that is, the time required for the first appearance of fecal pellets following the test meal, was prolonged 22 per cent.

The results obtained by the various workers on the effect of anoxia on

the motility of the small intestine are not unexpected. As far as it is known, the innervation of the small intestine is similar to that of the stomach and it is likely that anoxia would have similar effect on both organs; the difference, if any, would be one of degree.

No quantitative data could be found in the literature on the effect of anoxia on the intact colon alone. The colon has a different innervation from that of the stomach or small intestine and it would be unsafe to predict what effect anoxia would have on its movements. It is believed by some (14) that the blood supply of the colon is much less rich in oxygen than is that of the upper part of the gastro-intestinal tract. If this is true, it may be that the colon is relatively resistant to oxygen want. This, however, needs further study.

Since it is necessary to subject both man and animals to a pronounced degree of anoxia before the movements of the stomach are significantly affected, it may be inferred that the nausea and vomiting produced by high altitudes are not due to a direct effect on the stomach. It is known that some people suffer from marked mountain sickness at an altitude of 10,000 feet. At this altitude no pronounced change is noted in gastric motility.

Aviators often state that flying at high altitudes causes them to be hungry. It may be that these men confuse hunger with appetite. It is, of course, possible that in man certain altitudes may cause slight stimulation of hunger contractions. The carefully controlled work on dogs, however, gave no indication of this.

As previously mentioned, studies on the effect of anoxia on gastric emptying show a good deal of individual variation in both man and dog. Individuals who are particularly sensitive to oxygen want would show a pronounced retardation of gastric emptying at the altitude, for example, that a transcontinental airplane flies, that is, about 12,000 feet. Less susceptible individuals would be much less affected.

It must be recognized, however, that a moderate diminution of gastric motility presumably is of little practical significance. Anoxia doubtless would cause cerebral manifestations and cardiac dysfunction before any significant impairment would be noted in the behavior of the stomach. Compared to the central nervous system, the stomach is relatively resistant to anoxia.

Anoxia and gastro-intestinal permeability. The work of Hamburger (18) on the intestine, Bainbridge (4) on the permeability of the visceral capillaries, Starr (38) on the kidney, Landis (22) on the capillaries of the mesentery of the frog, Magee and Macleod (23) on

the intestine, and Van Liere and his co-workers (45) also on the intestine indicate that anoxia is capable of influencing the permeability of epithelium.

The difficulty with most of the work reported, however, is that the degree of anoxia was not measured. In some instances anoxia was produced by shutting off the blood supply or by producing injury to the epithelium by the application of corrosive chemical agents. Shutting off the blood supply produces a rapid and complete anoxia leading to irreparable injury in a short time; chemical injury to cells produces a dead membrane rather than a normal semi-permeable one. Conclusions drawn from experiments so performed must be accepted with some hesitation.

In order to avoid some of the criticism just offered, the following procedure has been used by the author and his co-workers in studying the effect of anoxia on absorption from the intestine of various substances. Matched pairs of dogs which had had no food 48 hours previous to the experiment were used. Sodium barbital (220 mgm. per kgm. body weight) given intravenously was used for an anesthetic. One animal served as a control and the other was subjected to anoxia. The small intestine was washed out with an isotonic solution of either sodium chloride or glucose heated to body temperature. With the exception of the duodenum, the entire small intestine was used as a loop. The loops were made of the same length by actual measurement. The substance to be studied was brought to body temperature before being placed in the intestine and was left in long enough so that at least 50 per cent was absorbed. Undue distention of the intestine was avoided. At the end of a given time the contents of the loops were removed and carefully measured and analyzed quantitatively for the substance in question. In nearly all the studies varying degrees of anoxia were used, ranging from a partial pressure of oxygen of 117 mm. Hg (8,000 ft.) to that of 53 mm. Hg (28,000 ft.) which partial pressure is about as low as is compatible with the life of a barbitalized animal over a period of two or three hours.

EFFECT OF ANOXIA ON ABSORPTION. *Absorption of water.* Bainbridge (4) in 1906 in explaining some of the factors concerning the flow of post mortem lymph suggested that lack of oxygen increased the permeability of visceral capillaries. It has been shown that the glomerular filtrate in a kidney with normal circulation is protein free (Wearn and Richard, 1924) (57). Starr (38) in 1926 showed, however, that a transient albuminuria not accompanied by any microscopically visible pathological

change could be produced by a stoppage or even a reduction of blood flow.

Landis (22) in 1928 studied the effects on capillary permeability of oxygen, high tensions of carbon dioxide and increased hydrogen ion concentration in single capillaries of the frog mesentery. He found that after a three minute period of oxygen lack, which he produced by compressing the mesenteric artery and vein, the permeability was so increased that the fluid in the capillary filtered through the wall at approximately four times its normal rate. Protein also passed through the capillary wall so that the effective osmotic pressure of the plasma proteins was reduced to one-half their normal value. As soon as the circulation was allowed to return, the capillary wall rapidly recovered its impermeability. When the mesentery was exposed to Ringer's solution completely saturated with carbon dioxide, the capillary permeability was slightly increased to fluid but remained normally impermeable to protein; one-half saturation of carbon dioxide had no effect. Within physiological limits an increase in hydrogen ion concentration produced practically no change in capillary permeability.

Van Liere, David and Lough (45) in 1935 reported studies on the effect of various degrees of anoxia on abosrption of water from the small intestine of barbitalized dogs, using the procedure previously outlined. They reported that absorption of water was not affected by anoxia until a partial pressure of oxygen of 80 mm. Hg (18,000 ft.) was reached. At this partial pressure of oxygen, considerably more water was absorbed by the anoxic dogs than by the controls. The results when statistically analyzed were highly significant. At partial pressures of oxygen of 63 and 53 mm. of Hg (24,000 and 28,000 ft.) the results, although not as striking, showed a definite trend in the same direction, that is, the dogs subjected to anoxia absorbed more water. These experiments were performed on a large number of dogs and it may be said then that the most rapid absorption of water from the small intestine of the mammal takes place at a partial pressure of oxygen of 80 mm. Hg. No adequate explanation can be offered for this rapid absorption of water at this particular pressure of oxygen. It is of interest to note, however, that this occurred at what Barcroft has termed the critical level of anoxia.

Van Liere, Northup and Sleeth (49) working with barbitalized dogs and using virtually the same technique as previously outlined, found that ephedrine administered either orally or intravenously had no effect on the intestinal absorption of water. When ephedrine was given intravenously during the period of absorption, the amount of water absorbed

from the small intestine was very slightly decreased. These findings are of interest since it was shown by Rudolf and Graham (32) that ephedrine produces vasoconstriction of the blood vessels of the intestines.

It has been shown (50) that in anemic anoxia less distilled water was absorbed from the small intestine of dogs which had suffered a hemorrhage of 3.2 per cent of their body weight. Since Wells (58) has shown that the absorbing force is proportional to the protein concentration of the blood, this decrease in absorption of water after hemorrhage was probably due to the fall of the blood plasma proteins. It is known, however, that distilled water is not a normal constituent of the small intestine and recently it has been shown by Dennis (12) that it is injurious to the epithelium of the ileum.

*Anoxia and gastric impermeability.* While anoxia may affect the permeability of the small intestine, no evidence has been presented to show that is affects the permeability of the stomach. A study has been reported (35) on the effect of severe degrees of anoxia on the permeability of gastric epithelium to water. The most severe degree of anoxia used in this study was a partial pressure of 53 mm. of Hg (28,000 ft.). Although animals were exposed to this degree of oxygen want for an hour or more, no appreciable influence on the absorption of water was found.

*Absorption of sodium chloride.* Hamburger (18) in 1896 reported that absorption of salt solutions in the intestine of dogs dead from one to twenty-four hours proceeded in the same manner as in living dogs. Magee and Macleod (23) in 1929 found that the walls of segments of gut became more permeable to solutions of sugars and electrolytes after devitalization.

Van Liere and Sleeth (51), working with barbitalized dogs and using the same technique as the senior author and his co-workers used with water, found that anoxia decreased somewhat the amount of absorption of physiological salt solution from the small intestine. Relatively mild degrees of anoxia (partial pressure of oxygen of 117 mm. Hg —8,000 ft.) caused a decrease in the amount of absorption. The absorption of fluid and the actual sodium chloride absorption ran parallel. The authors concluded from their work that oxygen aids in the absorption of physiological sodium chloride solution from the small intestine.

The author and his co-workers (49), using the same technique as just described, found that ephedrine administered either orally or intravenously had no effect on the intestinal absorption of isotonic sodium

chloride solution. In a subsequent paper (50) they reported that dogs which had been bled 3.2 per cent of their body weight absorbed more physiological salt solution from the small intestine than did control dogs. The results obtained with anemic anoxia, therefore, were different from those obtained with anoxic anoxia. The explanation offered was that after a severe hemorrhage there is a depletion of chlorides throughout the tissues of the body so that when the physiologic salt solution was placed in the intestine there was a higher diffusion gradient and the salt passed into the blood stream more rapidly.

*Absorption of sodium chloride in the presence of a sulphate radical.* It is known that the chloride ion is absorbed much more readily from the intestine in the presence of the sulphate radical (17). The mechanism of this is not understood. Van Liere and Vaughan (54), working with barbitalized dogs, studied the effect of various degrees of anoxia on the absorption from the small intestine of a solution consisting of equal parts of isotonic sodium chloride and isotonic sodium sulphate solutions. Anoxia depressed somewhat the absorption of the fluid and the sodium chloride; it did not, however, prevent the facilitation of the absorption of the chloride ion due to the presence of the sulphate radical. Anoxia had no effect on the absorption of sodium sulphate.

*Absorption of the sulphate radical.* Northup and Van Liere (28), working with barbitalized dogs and using degrees of anoxia ranging from a partial pressure of oxygen of 117 mm. Hg (8,000 ft.) to a partial pressure of 53 mm. Hg (28,000 ft.), reported that the absorption of an isotonic solution of magnesium sulphate was not significantly affected. As previously mentioned, neither does anoxia have any appreciable influence on the absorption of sodium sulphate.

These studies indicate that even severe degrees of anoxia do not alter the permeability of the intestine to the sulphate radical. This is of some practical importance since magnesium sulphate in many instances is given to patients with cardiac disorders which are often associated with anoxic states.

*Absorption of glucose.* It has been shown by Gellhorn and Northup (15) that circulatory factors can affect glucose absorption but the effects are inconstant and therefore in a large series can be ignored. Northup and Van Liere (unpublished work) found that degrees of anoxia up to a partial pressure of oxygen of 63 mm. of Hg did not influence the absorption of isotonic glucose solution. When lower partial pressures of oxygen were used, however, glucose was absorbed more rapidly.

It has been shown (49) that ephedrine (which is known to cause

vasoconstriction of the splanchnic region) either given orally or intravenously did not influence glucose absorption. It also has been shown that anemic anoxia, produced by bleeding dogs 3.2 per cent of their body weight, had no effect on the absorption of isotonic solution from the small intestine. The fact that the absorption of glucose apparently involves a phosphorylization process rather than an oxidative one (Verzar and McDougal, 55) probably explains why no significant change is produced by moderate and even moderately severe degrees of anoxia. The increase of absorption of glucose produced by severe degrees of anoxia (partial pressure of oxygen of 53 mm. of Hg or lower), however, can best be explained by the increased permeability produced by the severe degree of anoxia.

*Absorption of glucosides.* Anoxia in ranges of partial pressures of 93 mm. Hg to 53 mm. Hg has been found to have no effect either on the absorption of digitalis *per se* or on the fluid menstruum in barbitalized cats (46).

*Absorption of amino acids.* Northup and Van Liere (unpublished work) found that the absorption of glycine was increased by moderately severe degrees of anoxia (partial pressures of oxygen from 80 to 63 mm. Hg) but at more severe degrees of anoxia absorption was markedly decreased.

Studies on the effect of anoxia on absorption show that the amount of interference with absorption is presumably of no practical significance and that ranges of anoxia compatible with life would not interfere, so far as absorption is concerned, with the proper nourishment of the body. Whether the increased permeability often brought about by severe degrees of anoxia would allow certain split-products of proteins to be absorbed which would cause allergic manifestations or other untoward effects remains to be proven.

The main value of these studies in absorption is the light they throw on the mechanism of absorptive processes and especially that of the rôle of physiological oxidations. The studies on glucose absorption has lent support, for example, to the theory that the mechanism of glucose absorption is a phosphorylization process rather than an oxidative one since its absorption is not decreased by anoxia. Similar studies with other substances have shown how anoxia may affect the permeability of the epithelium.

Effect of anemic anoxia on gastric secretion. In 1936 Alvarez and Vanzant (2) reported a study on a large number of human beings concerning the relation between the hemoglobin content of the blood

and free gastric acidity. They found that the mean gastric acidity fell off sharply and the incidence of achlorhydria rose rapidly when the hemoglobin fell below 12 grams corresponding to 72 per cent. From their work they felt there was evidence to indicate that loss of blood in both animals and man can temporarily lower gastric acidity.

Clinical studies in cases of idiopathic microcytic anemia were reported by Hartfall and Witts (19) in 1933. In a series of 137 cases, 80 per cent gave evidence of achlorhydria; these investigators did not feel, however, that the low acidity was secondary to the anemia. Goldhamer (16), working with patients suffering from pernicious anemia, found a direct relation between the erythrocyte count and the amount of gastric juice secreted in a given time.

Apperly and Cary (3) in 1936 showed experimentally that free acid disappeared from the stomach when the red cell count of the blood fell below a certain critical level.

It will be seen when anoxic anoxia is discussed that anemic anoxia has a more profound effect on depressing gastric juice than has the anoxic type. One reason for this may be the loss of chlorides during hemorrhage.

EFFECT OF ANOXIC ANOXIA ON SECRETION. *Effect on gastric secretion.* Bayeux (5) in 1911, working with dogs, showed that at an altitude of 14,000 feet the volume of gastric juice was diminished but that the titratable acidity was not affected. Delrue (11) in 1934 transported dogs from his laboratory to higher altitudes and reported that at an altitude of 8,000 feet the gastric juice showed a decrease in pH and in titratable acidity. Hellebrandt, Brogdon, and Hoopes (20) in 1935, using human beings and producing anoxia for short periods by use of a rebreathing apparatus, reported that anoxia of the precoma type caused no appreciable decrease in gastric acidity.

Sleeth and Van Liere (34) in 1936, working on the response of the stomach to water in barbitalized dogs, found that only after a partial pressure of oxygen of 53 mm. Hg (28,000 ft.) had been reached was there a definite diminution in the titratable acidity and in the chlorides. In 1939 Pickett and Van Liere (31) subjected Pavlov pouch and Heidenhain pouch dogs to various degrees of anoxia; the most severe degree used was a partial pressure of oxygen of 63 mm. Hg (24,000 ft.). They reported that anoxia caused a decrease in the volume of gastric juice in all the dogs and the more severe the degree of anoxia, the greater the decrease in gastric secretion. The pH of the gastric juice did not change in the Pavlov group until a partial pressure of 63 mm. Hg was reached,

but the Heidenhain group was affected at 80 mm. Hg (18,000 ft.). On the other hand, the titratable acidity in the Pavlov group was decreased at a partial pressure of oxygen of 80 mm. Hg and that of the Heidenhain group at a partial pressure of oxygen of 63 mm. Hg, that is, just reverse of the effects produced on the pH. Total chlorides in the Pavlov group were not influenced by the ranges of anoxia used; in the Heidenhain group, however, they were decreased at a partial pressure of oxygen of 94 mm. Hg (14,000 ft.). The authors concluded from their work that the Heidenhain group was affected by less severe degrees of anoxia than the Pavlov group.

*Effect on intestinal secretion.* Northup and Van Liere (39) in 1939 studied the effect of anoxia on intestinal secretion in barbitalized dogs. Intestinal secretion was stimulated by injecting a peptone extract prepared according to the method described by Nasset and Pierce (24). They concluded that the intestine had a low energy requirement for secretion since it was only when a partial pressure of oxygen of 53 mm. Hg (28,000 ft.) was reached that a slight depression of secretion occurred. This depression in secretion, moreover, was not statistically significant even though 15 animals were used.

These studies on the effect of anoxia on secretion apparently show that the gastro-intestinal tract has a low energy requirement for secretion, since it is capable of withstanding relatively severe grades of anoxia before it is significantly affected.

This brief resumé indicates the relative paucity of our knowledge concerning the effect of anoxia on the gastro-intestinal tract. While indeed a good deal more work is needed on the effect of oxygen want on the entire gastroenteric tract, the fields which appear particularly promising for fruitful research are those which have to do with the effect of anoxia on secretion and absorption.

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# CARDIAC AUTOMATICITY AND RESPONSE TO BLOOD PRESSURE RAISING AGENTS DURING INHALATION ANESTHESIA

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Ever since the discovery of anesthetic agents their action on the circulatory system has been a subject of great medical concern. In this review we shall deal with the inhalation anesthetics, chloroform, ether and cyclopropane, and their actions on the automatic properties of the heart. Particular attention will also be paid to the cardiac effects of the sympathomimetic amines administered during anesthesia.

By automaticity of the heart is meant the production of the inner stimulus, the spread of the excitation and the rhythm of the resulting contraction. While the heart is automatic in the sense that it continues beating for a time when separated from either its blood supply or the central nervous system, it is of course normally subject both to humoral and nervous influences. The anesthetics as well as the sympathomimetic amines may not only affect the heart directly but also indirectly by way of these humoral and nervous mechanisms. The more important of these are concerned with the activities of the cardiac medullary and mid-brain centers, reflexes originating in the carotid sinus and aortic arch and variations in the secretory activity of the adrenal medulla.

It would be possible to outline the review on the basis of those physiological properties of the circulating system with which we shall be concerned. Most workers in the field however think in terms of a given anesthetic, and for this reason each agent will be taken up separately. Of the known gaseous anesthetics chloroform has been the most thoroughly studied and a knowledge of its circulatory effects will also serve as a background for the entire group.

Chloroform. The early work on chloroform had to do chiefly with the cause of the deaths which occasionally occurred during its administration. From clinical and physiological investigations a great diversity of opinion resulted as to whether the system most concerned

was the respiratory, cardiac or vasomotor. A great deal of this literature previous to 1900 has now mostly an historical interest. Of these early contributions only those need be cited which have pointed the way for later investigations. The older literature may be found in Heffter's Handbuch (82), Killian's Narkose (78) and in the standard pharmacologies.

*Effect of chloroform on the heart rate.* The action of chloroform on the rate of cardiac contraction is somewhat variable, depending on the way in which the experiments are carried out, the species of animal used, the dosage employed and the length of time the heart has been subjected to the anesthetic. It may be stated in general that no matter how the heart may be exposed to the agent the rate decreases with increasing concentrations and duration of the experiment.

Diastolic standstill has been described by Bandler (4) for the heart of Daphnia, by Pickering (114) for the chick embryo and by Carlson (28) and Meek (103) for Limulus. For the frog heart Dieballa (34) found that 0.126 per cent of chloroform in a saline solution produced cardiac standstill. Although weaker concentrations did not always slow the heart he never saw stimulation at any stage. Schmiedeberg (139) made similar observations on the frog heart as did Rasche (116). Kevdin and Trofimov (77) also produced cardiac standstill in the frog and found that $CaCl_2$ quickly restored activity. Of all the workers Hahn (63) is the only one apparently who reported acceleration of the frog heart with chloroform. She perfused the heart with 2.5 to 5 parts of chloroform per liter in calf's blood or serum.

For the turtle heart Vernon (164) observed that cardiac slowing is almost proportional to the chloroform concentration.

Excised mammalian hearts may always be finally arrested in diastole by sufficient chloroform in the perfusion fluid. However, for the dog heart isolated from the central nervous system by the method of Hering, Embley (45) noted no marked or abrupt changes in rate before the final stages of paralysis Sherrington and Sowton (150) stated that there was no alteration in frequency of rhythm incident to perfusion of the cat heart with low concentrations of chloroform in Ringer's solution. Chloroform in blood was much less effective than in saline or Locke's solution. Tunnicliffe and Rosenheim (161) reduced the rate of the heart by perfusing a quantity of fluid containing only 0.12 mgm. of chloroform. Using the Langendorff perfusion technique, Loeb (93) secured standstill in cat hearts with an 0.084 per cent chloroform solution. Winter (167) made similar observations. Rasche (116) ob-

served only a negative chronotropic effect when perfusing the mammalian heart.

With inhalation of chloroform several observers have described cardiac acceleration during the stage of excitement; Bock (20) for rabbits; MacWilliam (96) for cats and Cluzet and Petzetakis (31) for dogs. Halsey, Reynolds and Blackberg (64) also noted acceleration in dogs with doses so small that the reflexes still persisted. On the other hand L. Hill (73) has referred to cardiac slowing during the early stage of induction. According to Tiemann (154) light anesthesia may be borne by cats for an hour or more with no change in rate. For dogs on 2 per cent chloroform in air Embley (46) described a slow rate which might be prevented by vagotomy. Bradycardia was the sole change in rate described by Gaskell and Shore (54) for rabbits, by Hecht and Nobel (66) for dogs, rabbits and an ape and by Frommel (51) for guinea pigs. Beattie, Brow and Long (13) found that the normal rate of sinus discharge in cats dropped only slightly during the course of a 2 per cent chloroform anesthesia. With concentrations greater than 3 per cent the rate dropped steadily to as low as 120 per minute. In man the Glasgow Commission recorded slow pulses in 50 patients. Cluzet and Tixier (32) reported bradycardia in all 6 cases studied. I. G. W. Hill (72) found only three cases of bradycardia and one of acceleration in 15 clinical anesthesias but Steinfeldt (152) reported cardiac slowing in each of 8 patients studied.

The effect of chloroform on the heart rate in the early stages of anesthesia is then variable. The accelerations have doubtless been due to muscular movement and sensory stimuli of various kinds beyond the experimenter's control. The inhibitions of rate are also most likely examples of the well known vagal reflexes initiated by stimulation of the respiratory passages. Frommel (51) found that the bradycardia was reduced by vagotomy, atropinization or administration of the vapour by tracheotomy. Embley (46) also noticed the slowing was abolished by section of the vagi. In the dog and man these vagal reflexes are not easily induced, but Arthus (3) has described them in the rabbit when the nasal mucosa was stimulated by chloroform itself.

After anesthesia is established the reduced pulse rate persists or appears if there has first been acceleration. On this all workers agree. The slowing increases with the increasing depth of the narcosis until there is cardiac standstill. In these later stages the slowing cannot of course be reflex in origin and there is evidence both by Elfstrand (42) and Embley (46) that the vagi have lost much or all of their irritability.

Cardiac slowing in the later stages of the anesthesia must then be due to the direct effect of the chloroform on the heart or to indirect effects from the failing circulation.

*Cardiac irregularities in chloroform anesthesia.* Early observations both in the clinic and in the laboratory indicated that chloroform induced cardiac irregularities in addition to the changes in rate already mentioned. Actual stoppage of the heart which will be discussed in the next section was of course the most noticeable disturbance that could occur. Variations in conduction and rhythm were however evident though they could not be analyzed to advantage from the commonly noted pulse or blood pressure tracings. Advances were made possible by employing Engelmann's suspension methods and the venous pulse, but even then there could be no assurance of exactly what was happening until the development of electrocardiography.

Although Knoll (80) as early as 1878 had noted arrhythmia under chloroform and MacWilliam (96) in 1899 had described changes in irritability often in the form of a bigeminus, it was Levy and Lewis (92) who first showed by means of electrocardiograms that chloroform anesthesia may produce a series of irregularities from automatic centers in the ventricles ranging all the way from isolated extrasystoles to ventricular fibrillation. Hecht and Nobel (66) noted various disturbances in 15 dogs, 15 rabbits and 1 ape sometimes early but usually in the later stages of deep anesthesia. The P and T waves tended to be reduced in size and diphasic, the latter change being attributed to vagal influences. R was smaller, widened and notched. Auricular fibrillation was observed three times, once with an independent ventricular rhythm. Auricular extrasystoles were seen but once and ventricular twice. Only once did a group of ventricular extrasystoles lead into a tachycardia. There was but one spontaneous ventricular fibrillation although two appeared after massage in cardiac standstill. On the whole the irregularities, while present, were not as significant as those described by Levy and Lewis (92).

Nobel and Rothberger (108) were unable to demonstrate any arrhythmia in tracheotomized cats and dogs under light chloroform anesthesia (0.5–1 per cent), which seems at variance with the results of many other workers. In untracheotomized cats, however, irregularities appeared. In one animal subjected to 0.5 per cent chloroform scattered ventricular premature beats appeared. In another animal, first given 2 per cent vapour which was then lowered to 0.5 per cent, complete dissociation with a left sided ventricular autonomy was produced. In chloroformed

dogs Cluzet and Petzetakis (31) described lengthened P-R intervals and partial A-V blocks. Often there were long ventricular pauses broken by abnormal ventricular complexes. In prolonged anesthesia, negative P waves might appear and both P and T might become diphasic.

Frommel (51) working with guinea pigs found that the initial bradycardia of chloroform narcosis might pass into A-V block, complete but transitory. Instead of block there might be a salvo of extrasystoles. If the animals were allowed to succumb from the chloroform, the fatal termination might be a complete and permanent block or a decrease in both auricular and ventricular complexes passing finally into flutter and fibrillation. Following Levy's lead, Tiemann (154) studied cats in both light and deep chloroform anesthesia. In light anesthesia there were few irregularities unless preceded by a long period of deep narcosis. Deep anesthesia seemed to sensitize the heart to a succeeding lighter stage. In an anesthesia produced by chloroform of more than 1 per cent concentration, extrasystoles, nodal and particularly ventricular, were frequent. Light anesthesia alone was often endured for an hour or more with no change in rhythm. There were no changes in the P-R interval or in the shape of the regular waves of the electrocardiogram.

Eismayer and Wachsmuth (41) described an increase in respiratory arrhythmia at the beginning of narcosis which might lead to the development of automatic tertiary centers. A-V conduction disturbances were observed but the most significant change in the electrocardiogram was deformation of QRS complexes which was believed to indicate myocardial damage. Beattie, Brow and Long (12) state that with chloroform vapour of less than 2 per cent, cardiac irregularities do not appear even after 2 to 3 hours. With greater concentrations, however, the A-V conduction time lengthens and spontaneous abnormal QRS complexes occur, single, alternating with regular beats or in tachycardial groups. The T wave did not change unless the anesthesia was deep or the oxygen saturation of the blood seriously lowered.

Recently Chiarello (30) failed to observe arrhythmia in dogs during chloroform anesthesia. Oscillations in amplitude of the electrocardiographic waves, elevation of the S-T segment in leads I and II and inversion of T waves, were observed. The variations were believed to be due to transitory changes in the nutritive conditions of the heart and to be of no very great significance.

Studies of cardiac behavior under chloroform anesthesia have not been frequent in man. Cluzet and Tixier (32) in six clinical cases observed bradycardia and a slight reduction in amplitude of the waves of

the electrocardiogram. In only two of the six cases were there frequent extrasystoles. Hill (72) in fifteen cases found four unchanged under chloroform, three showed only minor variations, while the remaining eight exhibited marked disturbances, particularly extrasystoles of ventricular origin, one of which passed into a paroxysmal tachycardia. The disturbances generally disappeared as the anesthesia deepened. Six patients under chloroform were studied by Kurtz, Bennett and Shapiro (83). Four of these showed displaced pacemaker and two, extrasystoles.

Several attempts have been made to find the responsibility of the surgical procedures for the appearance of these irregularities. Although Rehn (118) thought the disturbances in clinical anesthesia were mostly nervous, due to reflexes from the operative field, Wachsmuth and Eismayer (165), Hill (72) and Maber, Crittenden and Shapiro (98) all agreed that the irregularities were a function of the anesthetic rather than of reflexes from the operative field.

From the above it is evident that cardiac irregularities are frequently observed in chloroform anesthesia, their occurrence depending chiefly on dosage, the species being studied and methods of administration. Most of the arrhythmias are reversible and disappear at once as the anesthesia is lightened and the animal regains consciousness. The literature so far cited refers to examples of smooth or what may be called normal chloroform anesthesia in which the administration of the anesthetic is skillfully carried out and all other conditions carefully controlled. In such cases though cardiac irregularities may occur, the anesthesia may be carried even to the paralytic stages of respiratory arrest while the heart continues to beat actively. At other times there are sudden circulatory collapses, the nature of which has given rise to a vast clinical and experimental literature.

*Chloroform anesthesia and circulatory collapse.* The problem of chloroform syncope came to the physiologists from the clinicians. Hardly had the agent been introduced before notices of sudden death under its influence began to appear. An interesting account of the early use of chloroform and ether in America has been gathered together by Hoff (74). In England the Registrar-General for 1890 reported thirty-six deaths from chloroform anesthesia. In 1899 (40) eighty-three deaths were reported and doubtless this was only part of the total.

The early studies of chloroform anesthesia had to do chiefly with the cause of these deaths. From clinical and physiological investiga-

tions a great diversity of opinion arose as to whether the system most concerned was the respiratory, vasomotor or cardiac. A great deal of fundamental work was developed in answer to this question.

Wood (168), MacWilliam (95), Gaskell and Shore (54, 55) and others clearly demonstrated that chloroform had a definitely poisonous effect on heart muscle and that death might be cardiac. But this by no means cleared up the mechanism of chloroform syncopeal death. Overdosage and myocardial paralysis did not seem to be determining factors, for in the clinics deaths were reported before the blood content of chloroform could apparently have become high enough to cause cardiac paralysis. The heart often stopped without gradually weakening, and if there was recovery, the beats were at once full and strong. Furthermore the deaths reported were usually in the early stages of anesthesia. During 1899 of the eighty-three deaths reported in England sixty-nine occurred before the operations were even started.

To explain the early deaths under chloroform anesthesia, Embley (45) (46) proposed vagal inhibition. In morphinized dogs by increasing the chloroform in the inspired air from 2 to 4 per cent, he found the beat was slowed or frequently suddenly arrested. These results were not prevented by section of reflex sensory paths from the respiratory organs but they were abolished by vagotomy or injection of atropine. Embley's idea was that chloroform raised the excitability of the vagus center, particularly in the early stages of the anesthesia, and that the inhibitory action was more intense because the poisoning of the heart muscle in some way rendered it more sensitive to the vagal stimulation. Embley's work was valuable in that it emphasized the importance of studying specific physiological mechanisms and in that it postulated an enhanced cardiac sensitivity. His use of morphine was undesirable. Although later workers found temporary stoppage of the heart in the early stages of chloroform anesthesia and that these might be removed by cutting the vagi (11), permanent inhibition could not be demonstrated. Probably some of Embley's inhibitions were really ventricular tachycardia or fibrillation.

The next attempt to explain cardiac syncope under chloroform was made by Levy (86, 87, 88, 89, 90, 91) whose ideas have markedly influenced all subsequent studies on the subject. In cats inhaling chloroform he noted that the blood pressure would often suddenly fall to zero and the animals die. On opening the chest the hearts were seen to be dilated and the ventricles in a state of fibrillation. At first he related these mishaps to the increased cardiac irritability resulting

from light anesthesia. Later (91) it became evident that the heart could be maintained beating at a perfectly regular rate even when lightly anesthetized, provided the anesthesia was level and unchanging, and no other disturbance was at work. The syncopes were conditioned by the change of cardiac state involved in the progress from deep to light anesthesia.

Working on the hypothesis that adrenalin might restore the circulation, Levy found on the contrary that doses as small as .03 mgm. were particularly efficacious in bringing on collapse. It is interesting that Oliver and Schaeffer (86) saw one case of collapse after injection of their adrenalin extracts into a chloroformed dog. Elliott (43) also described some accidents of the same nature but the anesthetic is not mentioned. Only MacWilliam (96) had previously noted any irregularities under chloroform.

In collaboration with Lewis (92), Levy investigated by means of the electrocardiograph the cardiac irregularities appearing under controlled concentrations of chloroform. In the cat after a few minutes' inhalation of 0.5 per cent chloroform ventricular extrasystoles regularly appeared. As the anesthetic increased to 1.5 and 2.0 per cent the irregularities disappeared but they returned as the anesthesia was lightened and often passed into a ventricular tachycardia. In deep anesthesia a dose of adrenalin as large as 0.065 mgm. induced only premature beats, but in light anesthesia as little as 0.016 mgm. caused ventricular tachycardia and fibrillation. Levy and Lewis believed that extrasystoles and ventricular tachycardia were stages on the way to ventricular fibrillation.

Independently Levy (86, 89) found that adrenalin produced fibrillation in light chloroform anesthesia after vagotomy, sympathectomy, pithing of the cord and in one case after the bulb was destroyed. It is notable that after pithing the cerebrum, 0.2 mgm. or nearly four times the usual dose of adrenalin caused only antecedent irregularities with momentary fibrillations. Stimulation of the stellate ganglia also precipitated fibrillation quite as well as the injection of adrenalin. Stimulation of the right accelerator mechanism was more effective than the left, later confirmed by Beritoff and Tschikamanauri (16), although Rothberger and Winterburg (130) had found that in the dog the left was more active in developing automatic ventricular centers. Under light anesthesia vagotomy was a good way to induce tachycardia which might proceed into fibrillation. This was easily explained on the assumption of an unrestrained sympathetic effect following the

loss of vagal tonus, but it was found that vagotomy was also effective following a previous section of the sympathetics which seemed to indicate that some intramuscular change of state was concerned. Heightened vagal tonus was not favorable for the development of fibrillation. Sensory stimulations of such nerves as the sciatic or splanchnics were followed by cardiac irregularities which occasionally led to ventricular fibrillation provided either the accelerator or adrenal nerves were intact.

Levy's ideas may be briefly stated as follows. Chloroform first renders the heart irritable, that is, liable to exhibit beats of heterogenic or ectopic origin. These actually occur only when the heart, sensitized by the chloroform, is subjected to some further exciting cause. Such simulations may be due to reflex increases of accelerator activity, reflex secretion of adrenalin, direct injection of adrenalin, or some change of state in the heart as exemplified by section of the vagi or varying the strength of the anesthetic, particularly from deep to light. Cardiac irritability is raised in light anesthesia and lowered in deep. Deep anesthesia, since the heart is depressed and not so irritable, is safer, but even light anesthesia if kept steady and not preceded by deeper stages (91) does not readily sensitize the heart. Sensitization is however readily induced by a change from deep to light anesthesia.

Levy did not believe in any over-dosage theory. His idea that cardiac irritability is greater under light chloroform has been borne out by the experiments of Tournade, Malmejac and Djourno (157) who showed that faradic stimulation produced fibrillation in light but not in deep anesthesia.

Shortly after Levy's series of papers, chloroform narcosis was again investigated by Nobel and Rothberger (108). Their findings in light chloroform anesthesia differed sharply from those of Levy in that they observed no irregularities, that injection of adrenalin with the exception of one fibrillation produced only extrasystolic rhythms, that section of the vagi decreased the effect of adrenalin and that sensory stimulation was ineffective in precipitating cardiac disturbances. These differences may have depended at least in part on different techniques, Nobel and Rothberger having introduced chloroform by way of tracheal cannulae into morphinized dogs. There was agreement on the general observation that adrenalin was more effective after light chloroform anesthesia than before.

While Nobel and Rothberger did not exactly oppose Levy's hypothesis that chloroform increases the irritability of the lower automatic

centers of the heart, they laid most emphasis on the vagus and accelerator mechanisms. Chloroform increased the activity of the cardio-inhibitory mechanism as shown by Embley (45), Schaeffer and Scharlieb (135, 136) and a number of still earlier workers. Adrenalin also stimulated the vagus and after its injection cardiac standstill of more than 4 seconds was observed. If the dose of adrenalin was small, the marked sinus bradycardia allowed the escape of impulses, mostly from the A-V node. With larger doses of adrenalin sympathetic stimulation of the ventricular structures began to appear. These automatic centers, not being normally under any vagal control, responded first with a few scattered beats which might rapidly increase, developing into a multifocal ventricular tachycardia or even a ventricular fibrillation. Such a conception was borne out by the experiments of Rotbberger and Winterberg (130) who brought on an A-V rhythm and occasionally fibrillation in the dog by simultaneous stimulation of the right vagus and left accelerator. Nobel and Rothberger's idea explained why cutting the vagi or administration of atropin rendered adrenalin less effective. Vagal stimulation was thereby eliminated, the sinus node was accelerated and to become dominant, ventricular automatic centers had to be stimulated to a much higher degree if ventricular tachycardia was to develop. van Dongen (37) has recently supported the same idea.

Both Levy and Nobel and Rothberger state that adrenalin injections in intact unnarcotized animals never led to cardiac irregularities, an observation out of harmony with the results of all recent workers. Nobel and Rothberger were the first who controlled all their experiments with electrocardiographic records. The observations of both these workers as well as most recent ones would be more significant and understandable if they had consistently given the weights of their animals, the doses of adrenalin in milligrams per kilo and the duration of all injections. Only on such a basis can comparisons really be made.

That ventricular fibrillation occurred in cats during the administration of chloroform as noted by MacWilliam and studied by Levy was confirmed by Embley (47) who observed it in about three of every five animals. He attributed it to an intermittent nervous control of the heart during the early anesthetic stages, marked reflex accelerator activity alternating with temporarily restored vagal control. In the dog an early chloroform syncope due to ventricular fibrillation was not observed by Embley (48) in several hundred experiments. If a collapse occurred it was a terminal process when blood pressure had fallen to a very low level. Syncope from chloroform in the dog seemed to be of

the vagal type only, being accompanied, however, by vasoconstrictor inhibition, vaso-dilator stimulation and myocardial depression. It was not easy to kill a dog with chloroform.

That there are definite species variations in susceptibility to chloroform and chloroform-adrenalin syncope is apparent from the literature so far cited. That chloroform did not strongly sensitize the dog heart to adrenalin was shown by Meek, Hathaway and Orth (105). Bardier and Stillmunkes (5) were unable to produce chloroform-adrenalin syncope in the frog, toad, eel, guinea pig or rabbit. Gautrelet and Halpern (56) also stated that the rabbit was refractory. It must be remembered however that some of these statements are apt to be misleading since they are based on only a limited study of dosages. It should be, but not always is, well recognized that the results depend in part on concentrations and duration of the injections. That dogs are much more resistant to chloroform than cats seems well established although Smirnow (151) inclined to the opposite view.

Bardier and Stillmunkes (8) produced chloroform-adrenalin syncope in dogs on doses of approximately 0.01 mgm. per kilo of adrenalin. It is to be noted that Meek, Hathaway and Orth (105) were unable to secure ventricular fibrillation with doses of adrenalin as small as this. Neither of these two groups of workers could demonstrate that the depth of the anesthesia was significant. Bardier and Stillmunkes agreed with Levy that the syncope was peripheral in origin and did not depend on the central nervous system. They believed that adrenalin served as a final stimulus to the heart already rendered irritable by chloroform, or that adrenalin suddenly reinforced the toxic action of the chloroform, an idea similar to that suggested by Richet. In a later publication (7) they strongly supported the latter view, though they presented no rigorous proof. In dogs they were unable to get ventricular fibrillation by stimulating the splanchnics, as Levy did in cats, and they found that there was actually a decrease in splanchnic irritability in chloroform anesthesia. For this reason they distinguished between chloroform syncope and chloroform-adrenalin syncope.

Smirnow (151) evidently produced chloroform-adrenalin fibrillation in dogs with ease but curiously not in cats under the same conditions. Tièmann (154) found that in cats cardiac irregularities were more common in deep than light chloroform anesthesia. He did confirm Levy in that ventricular fibrillation was most easily induced when the animals were carried from deep to light anesthesia. He found, however, under these conditions that fibrillation was not particularly frequent,

occurring in only four out of twenty experiments. Tiemann recognized two types of death from chloroform narcosis; one a diastolic standstill from overdosage and the other ventricular fibrillation.

Another interesting chapter on chloroform anesthesia was written by Beattie, Brow and Long (13, 23) who demonstrated that chloroform produced cardiac irregularities by virtue of its action on the hypothalamus. They found that in the cat anesthetized with 2 per cent or less of chloroform, extrasystoles occurred and could be maintained for long periods of time, provided the oxygen saturation of the blood and the respiratory exchange were kept constant. A Sherrington decerebration not only abolished any abnormal rhythm existing at the time but extrasystoles could not be produced later on inhalation of the optimum amount of chloroform vapour. It was therefore obvious that the region responsible for the arrhythmia was above the anterior colliculus. Its exact location was determined by appropriate transections and found to be in the hypothalamus, a few millimeters on each side of the mid line below the thalamic commissure. Direct stimulation of this region produced oxtrasystoles. Their production by stimulation of the hypothalamus was due first to stimulation of the general sympathetic pathway to the heart, as evidenced by their absence after extirpation of the stellate ganglia or section of the fibers supplying these ganglia, and second, to stimulation of the pathways controlling the secretion of adrenalin.

Under chloroform the stimulation of such nerves as the sciatic or radial called forth extrasystoles so long as either the sympathetic nerve connections to the heart or the adrenals were intact and the hypothalamus and thalamus were intact. Beattie, Brow and Long therefore concluded that the sensory side of the arc for the reflex secretion of adrenalin and for cardiac accelerator effects reached as high as the thalamus, from which direct connection was made to the hypothalamus. Although stimulation of the sciatic nerve did not call forth extrasystoles after midbrain transection it did cause a small rise in blood pressure. There were then synaptic centers below the hypothalamus which might still make the production of extrasystoles possible.

Many observations have been made on the relation of the cardiac nerves to chloroform and chloroform-adrenalin syncope, to some of which we have already referred. Nobel and Rothberger (108) got arrhythmia under chloroform only when the *vagi* were intact, but Beattie, Brow and Long (13) found that the spontaneous extrasystoles were not prevented by vagotomy. There is general agreement by

Levy (89), Bardier and Stillmunkes (5), Frommel (51), Heinekamp (67), Garrelon and Pascalis (52), Bouckaert and Heymans (21) and Bijlsma and van Dongen (19) that section of the vagi does not prevent chloroform-adrenalin fibrillation. Both Levy (89) and Bardier and Stillmunkes (5) have reported the chloroform-adrenalin response after the heart was deprived of all nervous connections, although it seems a larger dose of adrenalin was necessary.

According to Beattie, Brow and Long vagal stimulation abolished the extrasystoles from chloroform alone, and Levy (91), Embley (47) and Smirnow (151) have reported that vagal stimulation or increased vagal tonus as occurs with morphine, protected from the chloroform-adrenalin syncope. On the other hand Bardier and Stillmunkes (5) could not confirm such results, and Hill (72) and Parade (113) have reported the production of extrasystoles under chloroform in patients by mechanical stimulation of the vagus. Gautrelet and Halpern (56) have stated that vagomimetic drugs favor fibrillation.

That stimulation of the peripheral vagus might either cause or abolish cardiac irregularities has often been reported experimentally, but the explanations have always been disconcerting, especially in view of the generally accepted evidence that the vagus does not influence the ventricles directly. Scherf (137) on the basis of his studies with aconite, believed that the vagus freed chemical substances which increased the activity of ventricular centers. If one accepts the idea that irregularities may arise in different ways from the lower cardiac centers, some of the apparently contradictory results might possibly be explained. When single beats or even runs of tachycardia are regularly related or coupled to a normal one, they are not the simple expression of increased ventricular automatism but may be regarded as the rather passive response of a heterotopic center to a normal excitation wave which passes by. Such irregularities might easily be abolished or modified by vagal action. If the normal beats or rhythms originate independently from tertiary centers then the vagus would have no control over them. Since in the chloroform-adrenalin response there is a development of fibrillation through stages of extrasystoles and tachycardia, it might well be that the effect of vagus stimulation might vary, depending on the mechanism responsible at the moment for the irregularities. Goldberg and Rothberger (57) and Schott (140) have presented work in support of such ideas.

Section of the accelerators did not prevent a chloroform-adrenalin syncope in the experiments of either Levy (89) or Bardier and Still-

munkes (5), although the reviewer has found that the dosage of adrenalin must be significantly increased. In view of the work of Beattie, Brow and Long this probably means that the heart is being sensitized in part by impulses reaching it from the higher mid-brain centers. Accelerator stimulation has been shown to produce fibrillation by Levy (89), Bardier and Stillmunkes (5), Tiemann (154), and Beritoff and Tschikamanauri (16). In the cat the right accelerator is the more effective while the reverse is true for the dog.

Bouckaert and Heymans (21) have reported that denervation of the carotid sinuses and section of the aortic fibers protected from a chloroform-adrenalin syncope. Their explanation was that the unopposed sympathetic nervous action on the heart and the extra secretion of the adrenals prevented cardiac dilatation during the hypertension induced by the injection of adrenalin and so prevented fibrillation. This rests on the assumption that only dilated hearts fibrillate.

Most workers have held to the view that chloroform raised the irritability of the ventricular tertiary centers to a point at which some further exciting cause, particularly adrenalin, might readily induce extrasystoles, tachycardia or fibrillation. Instead of looking on adrenalin as a simple stimulant Bardier and Stillmunkes (7) regarded it as an agent which suddenly increased the toxicity of the chloroform. In support of this idea they quoted Delhert who found that adrenalin reinforced the action of chloroform. Garrelon and Pascalis (52) also believed that since adrenalin sensitized the organism to all anesthetics, the chloroform-adrenalin syncope was only a chloroform syncope in which the toxic action of the chloroform was reinforced indirectly by the adrenalin. The mechanism of reinforcement consisted of the addition to the circulation of a sensitizing thyroid substance demonstrated by Garrelon and Santenoise (53), the secretion of which was induced by the increased vagal tonus due to the adrenalin. In agreement with this hypothesis Garrelon and Pascalis found that a high section of the vagus did abolish the chloroform-adrenalin syncope while a low one was ineffective. A more rigorous proof would have been to test the dosage necessary to produce syncope before and after thyroidectomy. Hermann (70) has also accepted the idea that the toxic effect of chloroform is reinforced by adrenalin.

In the opinion of the reviewer the evidence that adrenalin strongly excites the automatic ventricular tissue of a heart already rendered highly irritable by chloroform, is too strong to be disregarded. Almost countless references might be cited showing the stimulating effect of

adrenalin on cardiac tissue independent of other factors. Reed and Smith (117) produced arrhythmia in the denervated frog heart; Bourne (22) showed that adrenalin was favorable to the production of ectopic beats in the dog's heart from electrical stimulation; Gorski (58) found not only that adrenalin stimulated the excised cat's heart but that it was thereby sensitized to further doses; Rothberger and Sachs (129) have quite recently shown the rhythmicity of auricular strips after treatment with adrenalin; and finally Nathanson (107) has induced cardiac standstill in the human heart and then produced automaticity in lower centers by an injection of adrenalin. Since ephedrine was not effective the result was attributed not to secondary effects but to direct adrenalin action.

There is also at present no evidence that adrenalin or that other sympathomimetic drugs act on the heart by way of mid-brain sympathetic stimulation. Marazzi (100) recently reported such an action on the preganglionic mechanisms but he was unable to demonstrate an increased outflow below the ganglionic depression. Pitts, Larrabee and Bronk (115) stated orally in their recent report that they had observed no adrenalin stimulation of hypothalamic outflow.

Although there are now a great number of sympathomimetic drugs, few but adrenalin seem to have been investigated in relation to chloroform anesthesia. Novadrenalin was found to be active by Tournade and Raymond-Hamet (159). Many other substances, however, are known to be able to bring on a chloroform fibrillation: nicotine (6), lobelin (160), hordenin (158), anagyren (156) and genet (27). All of these but genet may be assumed to act by increasing the secretion of the adrenals.

Many efforts have been made to find substances or procedures which would reduce or entirely prevent chloroform and chloroform-adrenalin syncopes. Gunn and Martin (62) recommended the intrapericardial injection of adrenalin itself for chloroform standstill in the rabbit's heart. More recently Tournade, Malmejac and Djourno (157) reported recovery in the dog from chloroform syncope by the intravenous or intracardiac injection of adrenalin combined with massage and artificial respiration. Henrijean (69) also cited the restoration of independent sinus and ventricular rhythms from injections of adrenalin. It is evident that these authors were dealing not with fibrillation but with a simple toxic depression of the heart, a real chloroform syncope. Although Tournade, Malmejac and Djourno found the treatment effective in most cases about one in ten dogs developed fibrillation that

was a true chloroform-adrenalin syncope. This danger had previously been pointed out be Bardier and Stillmunkes (7). Douglas (39) found that a preliminary injection of adrenalin some three minutes before the administration of chloroform protected from a later adrenalin injection, but this could not be confirmed by Tournade and Malmejac (155). The experimental literature in general would strongly recommend great caution in the use of adrenalin in chloroform anesthesia.

On the basis that chloroform-adrenalin fibrillation is due to the abrupt rise in blood pressure, as advocated by Shen (146), a number of substances and procedures which smooth out the blood pressure rise are reported to be protective; administration of hordenin (158), dioxane derivatives (146, 148) yohimbine (146), section of the presso-sensitive nerves (21), extirpation of the lumbar sympathetics (112), anagyrin (156), viper and scorpion venoms (8), restriction of the vaso-constrictor area (111), extirpation of the spleen and pancreas (111) and blockage of the reticulo-endothelial system (163). Protection has also been reported from the administration of morphine (151), $CaCl_2$ (77) (151), chloralose (121) and salt solution (120). Tiemann (154) found a loss of irritability to chloroform after parathyroidectomy, Bardier and Stillmunkes reported that adrenalin might be used as a restorative in chloroform anesthesia after hemorrhage and Hoff and Nahum (75) found that acetyl B methylcholin abolished chloroform-adrenalin irregularities.

Probably the most interesting of the long list are the agents which decrease the excitability of the heart itself and so eliminate the adrenalin effect. Such an action was found by Bardier and Stillmunkes for quinine (5) and quinidine (9) when given orally. Novocain has been shown by van Dongen (37) to protect the heart from electrically produced flutter and Shen and Simon (149) have used it to protect dogs from a chloroform-adrenalin syncope. According to Hermann and Jourdan (71) cocain injected intramuscularly increased the resistance of chloroformed dogs some three or four times to adrenalin. Dikshit (35) has reported that sodium barbital abolishes the irregularities of chloroform and adrenalin, without eliminating the blood pressure rise and they concluded that the irregularities were not due to pressor changes. The barbital effect is attributed by Knoeffel (79) to a diminution in adrenalin liberated by an over active sympathetic nervous system.

The idea that the chief difficulty in chloroform-adrenalin fibrillation might be the inability of the heart to withstand the intracardiac strain

imposed by the high blood pressure did not escape Levy. He examined the theory with care but felt forced to abandon it (90). Several later workers have, however, returned to the idea of intracardiac strain. Heinekamp (67) was impressed with the dilatation of a heart weakened by chloroform and its consequent inability to meet its load, particularly when driven by adrenalin. Beattie, Brow and Long (13) did not deny that the rise in blood pressure might have some effect in predisposing the heart to produce extrasystoles. They confirmed the observations of Levy that lowering the venous inflow to the heart abolished arrhythmia. Bardier and Stillmunkes (5) on the other hand found that a falling pressure from an acute hemorrhage did not prevent adrenalin from causing fibrillation. There was, however, a rise at the time of injection though it did not exceed the normal before hemorrhage. Evidence has been offered by Dikshit (35) that sodium barbital abolishes chloroform-adrenalin irregularities by its action on a mid-brain center and that the rise of blood pressure in itself is not directly effective on the heart. In his adrenalin studies on rabbits, Allen (1) has reported that if the adrenalin rise of blood pressure is prevented by a stabilizer, arrhythmia does not appear, but complete denervation of the heart does not prevent it. He believed that a rise of blood pressure from any cause will produce ectopic beats. Bouckaert and Heymans (21) attributed the stimulus for chloroform-adrenalin syncope to some kind of a sudden and intense change in the tonus of the vegetative nervous system.

Recently Shen (146, 147) has presented considerable evidence in favor of the importance of the rise of arterial pressure in determining the production of chloroform-adrenalin and benzol-adrenalin fibrillation. In particular the fibrillation fails to occur when the hypertensive action of adrenalin is either suppressed or reversed by previous injections of such substances as the dioxane derivatives or yohimbine. However, since van Dongen (38) has shown that the dioxane F933 makes the heart more resistant to electrical stimulation it may be that the protective factor is the direct restraining action on the heart and not the hypotension. For benzol-adrenalin fibrillation Shen concludes that the heart is intoxicated by benzol, predisposed to arrhythmia by the adrenalin and abruptly excited by the sudden rise in pressure. The same idea would doubtless hold for chloroform.

It is probably impossible at present to evaluate the exact part played by blood pressure rises on cardiac arrhythmia under chloroform although it is doubtless often a factor in the picture. It is well recognized

that an increased intracardiac pressure within limits stimulates the muscle fibers to greater contraction and it might well be that it can act as a stimulus to a heart rendered hyper-irritable by chloroform.

In the present state of our knowledge it seems impossible to arrive at any analysis of the chloroform effect on the heart which disposes it to fibrillation in terms of fundamental muscular physiology. The work of Sasaki (134) who gave evidence of a shortened refractory period of cardiac muscle under chloroform has been repeatedly quoted in this connection. MacWilliam (97), however, has reported no change in the absolutely, but a lengthening of the relatively refractory period. Van Dongen (37) found that novocain protected from extrasystoles and fibrillation produced in cats by adrenalin or $BaCl_2$ but there were no changes in the refractory period or conduction time. Bijlsma and von Dongen (19) have stated that strophanthin which forms fibrillation lengthens the refractory period, while ether, under which fibrillation seldom occurs, shortens the refractory period. These results give some support to the theory that fibrillation may be a heterotopic rhythm of great frequency and that drugs favoring fibrillation do so by stimulating the ventricular automatic mechanisms.

ETHER. From the circulatory standpoint ether resembles chloroform although the differences in action from a quantitative point of view are extremely great. Ever since their introduction as anesthetics, ether has been recognized as much less toxic than chloroform. A comparison of the two agents may be found in Beecher's *Physiology of anesthesia* (14).

*The effect of ether on the heart rate and strength of contraction.* Reviews of the older literature may be found in Heffter (82) and Killian (78). For hearts of the chick embryo (114), Limulus (28), fish (15), and frog (34) there is general agreement that in physiological saline ether has little effect in concentrations under about 5 to 10 parts per thousand but above this both rate and amplitude decrease until there is cardiac standstill in diastole. An initial stimulation has been observed by some workers.

In the perfused warm-blooded hearts stimulation from ether in the perfusing fluid has been reported rarely if at all. Bock (20) noted only slight depression in a rabbit heart-lung preparation. Tunnicliffe and Rosenheim (161) found that 0.2 per cent did not stop the excised heart. Loeb (93) produced diastolic standstill in the cat's heart with 1.7 per cent ether in blood-saline. Derouaux (33), Schramm (141) and Sarter (133) all reported depression in rate and amplitude as the

concentrations increased. Burridge (24) has stated that ether like chloroform has a twofold effect on the heart, an immediate depression followed by augmentation when the drug is removed.

That inhalation of ether always leads to a fast heart has long been recognized both in the laboratory and the clinic. In dogs (31) this occurs in the stage of excitation and remains during the entire period of narcosis (165). In guinea pigs it may be preceded by a period of slowing (51). Transitory changes in the early stages are doubtless due to reflexes from the irritating effects of the gas. Many workers have attributed the rapid heart rate to impairment of the vagal mechanism. Thus Kobacker, Lester and Rigler (81) found that the vagus was non-responsive to stimulation in cats when the animals were completely anesthetized. The site of ether action was at the peripheral ganglia since the pre-paralytic stimulation of nicotine disappeared but acetylcholine remained effective. Shafer, Underwood and Gaynor (145) noted that vagal stimulation affected the heart rate much less in etherized dogs than it did in the same animals after decerebration. Elliott (44), Cattell (29) and Bhatia and Burn (18) all believed that adrenalin was liberated during ether anesthesia. Samaan (132) has made the most careful study of the mechanisms responsible for the increased ether heart rate. By elimination at appropriate times of the vagi, cervical sympathetics, buffer nerves, splanchnics and adrenals he came to the conclusion that the increase in cardiac rate depended on 1, paresis of the vagal inhibitory mechanisms; 2, augmentation of cardio-sympathetic impulses; and 3, liberation of certain sympathicomimetic hormones such as adrenalin or sympathin.

*Ether anesthesia and cardiac irregularities.* The early workers were so impressed with the serious cardiac disturbances under chloroform that those observed under ether seemed minor and unimportant. Not until the electrocardiograph came into use were the cardiac irregularities in etherized animals carefully studied. The waves of the electrocardiogram for regular cycles initiated by the sinus are not particularly modified during ether anesthesia in laboratory animals. Small variations in the P waves have been reported by Cluzet and Petzetakis (31), Bettlach (17) and Chiarello (30). These possibly indicate slight displacements of the normal pacemaker. Others have noted the disappearance of the P wave and have interpreted it as nodal rhythm although Henrijean (69) thought this irregularity was not characteristic of ether anesthesia, the A-V node being less susceptible to ether than chloroform. Changes in the QRS complex described by Miller and

Felberbaum (106), Bettlach (17) and Foa (49) are limited to minor variations in voltage. Variations in the T wave have been noted by numerous investigators; Miller and Felberbaum (106) in cats and Eismayer and Wachsmuth (41), Bettlach (17), Chiarello (30) and Foa (49) in dogs. The T wave changes were usually reversals in direction, deep negative waves being often observed and believed by Hecht and Nobel (66) to indicate vagal action. In view of the variability of the T waves, particularly in the electrocardiogram of dogs, as shown by Barnes and Mann (10) and Katz, Soskin and Frisch (76), the changes described above probably have little significance. It should be noted that the modifications of the electrocardiographic waves under ether are reversible and usually quickly disappear when the anesthesia is terminated. Routier (131) regarded them as variations within physiological limits.

Blocks and ectopic beats are rare in light ether anesthesia, but as narcosis deepens they may begin to appear. Striking as they may be, they rapidly disappear on removal of the ether. There is no tachycardia from secondary or tertiary centers. The absence of rapid ventricular rhythms is in sharp contrast to what occurs under chloroform. Details may be found in many of the references already given, but particularly in the papers of Frommel (51) and Miller and Felberbaum (106). The latter authors reported that in twelve deeply etherized dogs partial and complete A-V block occurred three times each, nodal rhythm three times, ventricular extrasystole once and auricular fibrillation and flutter once each. Brow, Long and Beattie (23) mentioned that ventricular extrasystoles are found under ether but not as many as under chloroform. Even if the ether was crowded to cardiac standstill Henrijean (69) observed that the EKGs of the last beats were of the normal type. Frommel (51) and Eismayer and Wachsmuth (41), however, found that as the respiratory pauses lengthened and the heart began to fail extrasystoles from secondary or tertiary centers appeared and finally passed into ventricular rhythms with bizarre QRS complexes. Such results are in agreement with the experience of most laboratory workers.

Studies of cardiac irregularities in clinical anesthesia with ether are not too numerous. The results on the whole agree with those already described for laboratory animals. Only Cluzet and Tixier (32) and Unger and May (162) found no modifications of the EKG or irregularities under ether. Heard and Straus (65) had one nodal rhythm in twenty-one cases, a rather low incidence for this type of abnormality. Levine (85) in eight cases observed auricular tachycardia twice and

auricular fibrillation once. Lennox, Graves and Levine (84), Marvin and Pastor (101) and Steinfeldt (152), in one-third to one-half of their cases have all described irregularities consisting of displacements of the cardiac pacemaker, auricular tachycardia, nodal rhythm and premature auricular and ventricular beats. Wachsmuth and Eismayer (165) noted similar irregularities which they attributed to the anesthetic and not to sensory impulses from the field of the operation as Rehn (118) had proposed. Hill (72) associated the irregularities with the stage of induction. He noted displacements of the pacemaker, variations in P-R intervals but only rarely extrasystoles or extrasystolic rhythms. Maher, Crittenden and Shapiro (98) observed nodal rhythm in thirty-two and QRS changes in fifteen of seventy ether cases. Deep anesthesia was the most important factor associated with these changes. They could not be related to surgical procedures and they all disappeared when the anesthesia was discontinued. Kurtz, Bennett and Shapiro (83) included twenty cases of ether anesthesia in their recent studies. Over 65 per cent of these showed downward displacements of the pacemaker while in only 20 per cent were extrasystoles observed. Extrasystoles were more frequent in patients with heart disease than in those with normal hearts. It is noteworthy that the extrasystoles arose in all cases from the auricle or A-V node and never from the ventricle.

From both the laboratory and the clinic it may be safely concluded that ether does somewhat favor certain forms of cardiac arrhythmia. These are mostly delays in A-V conduction, partial blocks, A-V rhythm and premature beats. Ventricular tachycardia and ventricular fibrillation are rarely if ever observed. For the most part the sinus pacemaker maintains its dominance as pointed out by Schlomovitz (138). The irregularities are most apt to occur early in induction or near the level of respiratory arrest, though they are to be observed in all stages. They are evidently not particularly related to operative procedures. The effects of increased $CO_2$ and $O_2$ deficiency have not been thoroughly studied. All the disturbances disappear on discontinuing the anesthetic.

*Ether and adrenalin.* Levy (90) was not able to produce fibrillation in cats under ether by injecting adrenalin, an observation confirmed later by Bardier and Stillmunkes (5) for dogs. The statement of Papilian, Cosma and Russu (110) that an adrenalin syncope cannot be produced in animals under ether is, however, too far reaching, for the results depend upon the dose of adrenalin administered. Nebel and Rothberger (108) secured a fast left sided ventricular tachycardia in the cat. Fibrillation may be produced in any animal, normal or anesthetized, if adrenalin is given in large enough quantities.

The exact amount of adrenalin or others of the sympathomimetic amines necessary to produce ventricular tachycardia or fibrillation in animals under ether has not been determined, a matter well worthy of investigation. That the definite dose is larger than for chloroform or cyclopropane has been clearly shown by Meek, Hathaway and Orth (105). This is not evidence, however, that ether has decreased the normal sensitivity of the automatic tissues to the drug. It may even have increased it but nothing like to the extent of what is found for chloroform or cyclopropane. Van Dongen (36) has recently reported that ether favors the fibrillation produced by electrical stimulation but that it has no effect on the development of the rhythms caused by $BaCl_2$ or adrenalin. Ether thus facilitated the formation of stimuli by electrical currents but had no effect on the formation of stimuli by chemical agents.

An ether-adrenalin syncope comparable to that of adrenalin and chloroform is thus not found either in the laboratory or the clinic. Fibrillation may obviously occur in ether anesthesia from other causes but any relation to the anesthetic has not yet been demonstrated.

Cyclopropane. The most recent inhalant to attain widespread popularity has been cyclopropane. Though first prepared by A. von Freund in 1882 (50) its anesthetic properties were not investigated until 1929 by Lucas and Henderson (94, 68). Waters and Schmidt (166) and Stiles, Neff, Rovenstine and Waters (153) introduced cyclopropane as a clinical anesthetic in 1934. The literature on all aspects of the new anesthetic is well covered in a recent monograph by Robbins (126).

*The effect of cyclopropane on heart rate and strength of contraction.* Waters and his associates (166, 142) stated in their early work that cyclopropane decreased the heart rate both in men and dogs. That the rate in dogs actually increases was shown by Meek, Hathaway and Orth (105) who recorded an increase in fourteen out of seventeen trained animals. Robbins and Baxter (123, 124, 125) have also found a greater heart rate in non-premedicated, untrained and trained dogs under cyclopropane anesthesia. Although the resting rate of the trained dogs was lower than the untrained the final rate in stage $III_{2-3}$ was the same for both groups, an average of 146 to 149 per minute. In man the heart rate is usually reduced below the resting level (143). Shackell and Blumenthal (144) have noted that Rhesus monkeys react like man and that the higher the concentration of cyclopropane the slower the rate.

The exact mechanism by which cyclopropane increases the heart

rate in dogs has not yet been carefully analyzed in the quantitative way that Samaan (132) has used for ether. That the cardio-inhibitory center is most concerned is evident, however, from work by Robbins, Fitzhugh and Baxter (128). They noted that removal of the sympathetic innervation of the heart in dogs had little effect on the resting rate. Furthermore this bilateral removal of the sympathetics under cyclopropane did not immediately alter the heart rate, as it should if the rate were being maintained by accelerator stimulation (128). There is no record of cutting the cardiac vagal fibers under cyclopropane to find the exact extent to which the anesthetic had decreased vagal tone. There can, however, be little doubt that this is the method of acceleration.

After premedication with morphine, however, Robbins and Baxter (125) noted that cyclopropane further decreased the already slow heart rate. In thirteen morphinized dogs the average rate was 73, which was reduced to 68 under cyclopropane anesthesia. Wherever the anatomical point of action of morphine may be, there has never been any question that it was on the vagal mechanism, and that the net result was an increase of inhibitory action on the normal pacemaker. If the action of cyclopropane is reversed after morphine it would seem to be an example of chemical rather than physiological synergism.

*Cardiac irregularities under cyclopropane.* It is common experience that non-premedicated animals may generally be anesthetized with cyclopropane and carried to the point of intercostal paralysis, stage $III_3$, without signs of cardiac arrhythmia or abnormal changes in the electrocardiogram. Seevers, Meek, Rovenstine and Stiles (142) reported that only three dogs out of twenty-seven showed extrasystoles before stage $III_3$ and recently Robbins and Baxter (125) found only one in forty. Up to this stage changes in the electrocardiogram are negligible. Bettlach (17) noted that negative T waves became positive in leads II and III but remained negative in lead I, while they were markedly deepened in lead IV. The records presented by Robbins have shown no details of interest.

Although as just stated an animal may be carried indefinitely in a moderate degree of anesthesia under cyclopropane without any signs of cardiac disturbances, that irregularities do occur under certain conditions is equally well known. As Beecher has stated, the ultimate value of cyclopropane may well depend on the evaluation of its cardiac effects.

In their first pharmacological studies of cyclopropane Lucas and Henderson (94) noticed irregularities on their blood pressure tracings

which appeared to be missed beats. These were more or less temporary and disappeared as the concentration of the gas was diminished. Waters also observed during preliminary clinical experiments that an occasional patient developed some cardiac arrhythmia if the concentration of the anesthetic was high. This led to a study of the respiratory and electrocardiographic changes in cyclopropane anesthesia by Seevers, Meek, Rovenstine and Stiles (142) in which it was shown that the irregularities which had been observed were not due to any contaminants such as propylene, but to the gas itself. These authors correlated the alveolar concentration of cyclopropane with the stages and planes of anesthesia first described by Guedel (60), respiratory rate and volume and cardiac rhythm. Surgical anesthesia in dogs was secured with a mixture of 33 per cent of cyclopropane in oxygen. Respiratory arrest appeared at 39 per cent. Premedication with morphine reduced these percentages. Later analyses by Robbins (122) on both mixture inspired and blood showed a remarkably close agreement with these findings.

Cardiac irregularities according to Seevers et al began to manifest themselves at about the time respiration ceased, although there was considerable variation and seven out of twenty-seven animals showed extrasystoles before respiratory paralysis. The types of arrhythmia were auriculo-ventricular block, nodal and ventricular extrasystoles, nodal and ventricular rhythms, ventricular tachycardia and auricular and ventricular fibrillation. With the exception of the two ventricular fibrillations the heart returned in all cases to its normal rhythm as the anesthesia was lightened or artificial respiration instituted.

Seevers and his collaborators noted that irregularities were abolished by atropine but that they reappeared on increasing the concentration of the anesthetic. They believed this indicated a vagal origin for the disturbances although it is more likely such an effect is only vagal indirectly since with the increased heart rate under atropine the sinus rate is so high the lower automatic centers cannot express themselves. These authors clearly recognized that oxygen lack was a factor in producing the irregularities as they disappeared on the addition of oxygen or institution of artificial respiration. However, since they reappeared at higher concentrations of cyclopropane under artificial respiration and since they observed two deaths by fibrillation at a time when alveolar oxygen was sufficiently high, they emphasized the toxicity of cyclopropane itself.

Robbins and Baxter (123) have confirmed the work of Seevers et al.

by making in addition analyses of the blood for oxygen, carbon dioxide and cyclopropane. In eleven animals they found that the concentration of cyclopropane in the arterial blood at the time of respiratory arrest was 33 mgm. per 100 cc. and that on the average 6.0 minutes later cardiac irregularities began to appear. Seven of the animals recovered on the institution of artificial respiration, and irregularities under this condition did not appear the second time until the concentration of cyclopropane in the blood reached 43.6 mgm. per 100 cc. Section of the vagi had little effect on these results. Arterial oxygen of course rapidly declined after respiratory arrest and at the time cardiac irregularities appeared it averaged only 2.8 volumes per cent.

Robbins and Baxter were quite naturally impressed with the low oxygen values present at the time arrhythmia appeared and knowing that Greene and Gilbert (59), Mathison (102) and Resnick (119) had demonstrated similar irregularities in oxygen want, they concluded that the cardiac irregularities developing during the period of respiratory arrest were not due to any effect of cyclopropane on the heart but to the extreme degree of anoxemia. It is the opinion of the reviewer that this conclusion is too sweeping since it has been observed by Robbins and Baxter, Seevers et al. and others that in both man and animals irregularities do occur occasionally or even frequently before there is any significant degree of anoxemia. That cyclopropane has affected the heart long before the stage of respiratory arrest is also clearly shown by the work of Meek, Hathaway and Orth (105) to be described shortly. Robbins agrees with Seevers et al. that in higher concentrations cyclopropane does have a direct effect. It would be curious if it had no effect in the lower dosages. However, that a decrease in the oxygen content of arterial blood accentuates the cardiac irregularities and may be the chief factor in bringing them on is a point well proven by Robbins and Baxter and it is of great practical importance.

That morphine administered before cyclopropane predisposes the hearts of dogs to irregularities such as A-V block, nodal rhythm and ventricular extrasystoles has been observed by Robbins, Fitzhugh and Baxter (128). It should be noted that the cases studied by Kurtz, Bennett and Shapiro (83) in which extrasystoles and multifocal ventricular tachycardia appeared, had all received morphine and scopolamine or atropine. Although the dose of morphine used clinically may not be large enough to be dangerous in this connection, its action under cyclopropane should be more carefully studied.

Both sodium barbital and amytal have been found by Robbins and Baxter (127, 125) to protect against the cardiac irregularities induced

by cyclopropane. Cardiac arrest occurred much later after respiratory arrest than in the controls; only two of fourteen dogs receiving the barbiturates showed irregularities; and the arterial content of oxygen was higher at the time of respiratory arrest. These results justify the author's suggestion that such premedication be tried in man.

Guedel (61) has recently reported protection from cyclopropane arrhythmias by evipal, pentothal and nembutal. Of these pentothal was about 90 per cent efficient but evipal seemed more desirable since it was 80 per cent efficient and longer lasting. Guedel believes that there is a definite arrhythmic range in cyclopropane anesthesia beginning at about the point of respiratory standstill and continuing until the concentration of the gas reaches 40 per cent or more. If the concentration of the cyclopropane is further increased by artificial respiration the irregularities disappear, but they reappear and are more marked on the ascent from the deep anesthesia.

*Effect of cyclopropane on the irritability of the automatic tissue.* It is not enough in judging an anesthetic merely to note that the heart shows no arrhythmia. The normal pacemaker might be approaching a stage of inhibition which would allow escape phenomena, or ectopic centers might be on the point of exhibiting activity, should an additional stimulus appear. This reasoning led Meek, Hathaway and Orth (105) to test the condition of the automatic tissue of the hearts in controlled stages of cyclopropane anesthesia by a standard injection of adrenalin. A dose of 0.01 mgm. per kilo in 5 cc. of normal saline was chosen and this was injected intravenously at the rate of 1 cc. per 10 seconds. While the standard injection of adrenalin in seventeen unanesthetized controls produced the usual number of extrasystoles which were interpreted as escape phenomena due to the slow sinus rate, there was only one example of ventricular tachycardia and ventricular fibrillation never appeared. Under light cyclopropane anesthesia adrenalin produced ventricular tachycardia in eleven of the seventeen animals and one succumbed to fibrillation. Under deep anesthesia sixteen of seventeen dogs showed ventricular tachycardia and one died of fibrillation. The tachycardias were multifocal in type. In light anesthesia their duration was 19 seconds and this was increased to 44.5 seconds in deep anesthesia. The effect was directly related to the depth of the anesthesia. These conclusions were confirmed in a later paper by Orth, Leigh, Mellish and Stutzman (109) who found in twenty animals that all showed ventricular tachycardia with adrenalin and cyclopropane and five succumbed to fibrillation.

Similar experiments with many of the same animals under chloroform

and ether demonstrated that in dogs cyclopropane had a more marked stimulating or sensitizing effect on the ventricular automatic tissue than either of the other agents.

Although these experiments showed beyond question that adrenalin was contraindicated in cyclopropane anesthesia they should not be taken to mean that cyclopropane is a particularly dangerous anesthetic. The irregularities with the exception of fibrillation are easily reversible and cyclopropane has the advantage over most anesthetics in that the tissues may be quickly desaturated. Danger may thus usually be quickly averted.

Since adrenalin injected during cyclopropane anesthesia resulted in serious cardiac irregularities, Orth, Leigh, Mellish and Stutzman (109) investigated the action of other blood pressure raising amines. A series of twenty dogs were injected with the standard dose of adrenalin for controls. All of these showed ventricular tachycardia and five ventricular fibrillation. By using doses approximately equivalent to 0.01 mgm. of adrenalin in blood pressure raising power they found that the following amines in cyclopropane anesthesia acted on the ventricular automatic tissue similarly to adrenalin; arternol, epinine, kephrine and cobefrine. By the same methods it was found that ephedrine, propadrine, benzedrine, paredrin, synephrine and neosynephrine did not exert any such cardiac effects. With the exception of noeosynephrine they did however markedly accelerate the sino-auricular rate. In the dog under cyclopropane, neosynephrine is the sympathomimetic amine most favorable to the heart.

There is a wide variety of side chain groupings in the above mentioned drugs. Adrenalin has a side chain of methylamine ethanol; arteronol has amino ethanol; epinine, methylaminc ethane; kephrine, methylamino keto ethane; and cobefrine, amino propanol. It seems however that these side chains must always be attached to a 3,4 di-hydroxy ring in order to be very effective. Agents with only one or no hydroxyl did not stimulate the heart sensitized by cyclopropane. The strong stimulating action of the amines with two hydroxyls was not seen under all anesthetics. In ether anesthesia neither ephedrine, arterenol, cobefrine or neosynephrine produced ventricular tachycardia. Under chloroform some cardiac sensitization was shown to cobefrine and arterenol, less to ephedrine and none to neosynephrine.

*Protection from the stimulating effects of sympathomimetic amines under cyclopropane.* It was shown by Meek and Seevers (104) that after premedication with sodium barbital there is a higher degree of

protection against the cardiac effect of at least one of the sympathomimetic drugs, ephedrine. This suggested to Orth et al. (109) that the cardiac action of blood pressure raising agents might be reduced under cyclopropane provided the animals were previously barbitalized. Intravenous injections of 200 to 225 mgm. of sodium barbital per kilogram, 50 mgm. of amytal per kilogram and 25 mgm. of nembutal were therefore made before induction. When the anesthetized animals were fully under the barbiturate, the test dose of adrenalin was given. No evidence of protection could be secured, both ventricular tachycardia and fibrillation frequently appearing. These results are not necessarily in contradiction to the protection from cyclopropane effects alone reported for barbiturates by Robbins and Baxter (125) since the heart was subjected to two sets of stimulating influences instead of one.

Recently Burstein and Marangoni (25) reported that 5 mgm. per kilo of procaine given either before adrenalin injections or during the stage of ventricular tachycardia reduced the incidence of ventricular fibrillation in dogs during cyclopropane anesthesia. These results have been confirmed by the reviewer. Since the injection of procaine solution into the circulation of man is frequently followed by untoward reactions, Burstein, Marangoni, De Graff and Rovenstine (26) (99) have recently investigated the protective action of less toxic substances of the same chemical group, para-amino benzoic acid, paramon and sodium para-amino benzoate. Administration of these drugs prior to a test dose of adrenalin during cyclopropane anesthesia reduced the incidence of ventricular fibrillation. These authors also found that the intracardiac injection of procaine at the time of fibrillation in dogs effected a return to normal in a number of cases. The other p-amino benzoic acid derivatives were not effective. A large number of pharmacological agents remain to be studied in this connection.

Allen, Stutzman and Meek (2) have recently shown that after decerebration in dogs under cyclopropane anesthesia the test dose of adrenalin no longer produced ventricular tachycardia. The integrity of some center above the pons was thus necessary for a cyclopropane-adrenalin response. Lesions of the pons at the level of the fifth nerve, removal of the stellates with all connections from the upper fifth thoracic ganglia and the intravenous injection of ergotamin also abolished the response. The authors concluded that cyclopropane sensitization of the heart took place because the anesthetic stimulated a mid-brain center which then sent impulses to the heart by sympathetic pathways. The direct action of adrenalin on the heart thus sensitized produced the ventricular

tachycardia. The mechanism of action for cyclopropane was thus shown to be similar to that described by Beattie, Brow and Long (13) for chloroform. No evidence for mid-brain sympathetic stimulation of adrenalin could be found by Allen, Stutzman and Meek.

In decerebrated dogs under cyclopropane anesthesia abrupt rises in blood pressure may still be produced by the sympathetic amines without the appearance of ventricular tachycardia or fibrillation according to Allen, Stutzman and Meek. These results are at variance with the ideas of Shen et al. (148) who have postulated the height or abruptness of the rise in blood pressure as the cause of chloroform-adrenalin syncope.

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# PHYSIOLOGY OF ITCHING

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"Itching is an unpleasant cutaneous sensation which provokes the desire to scratch." This trivial definition, about 200 years old (32), has not been modified in spite of advancing knowledge. It has been used to differentiate itching from other sensations which at times have been compared or confused with itching although they do not provoke the desire to scratch. Thus the sensation of vibration which is a function of the touch sense and is elicited by touch stimuli of high frequency has nothing in common with itching (22). Neither has the "formication" or "prickling" sensation which is perceived in circulatory arrest of the limbs, and which, according to recent research, arises from deep seated receptor organs under the skin (20).

"Superficial tickling" however seems to correspond with the above definition. If a light hair is drawn across the skin or neighboring mucous membranes, the sensation will be called a tickle, nevertheless in most persons it leads to the motor response of rubbing or scratching (55). For many years superficial tickling was regarded as a sensation different from itching (38, 71, 24), particularly after von Frey advanced experimental data supporting the assumption that tickling is elicited by stimulation of tactile receptors if the touch stimulus is weak, small and wandering, whereas itching is a function of the pain sense (24, 25). However, von Frey himself pointed out that by moderately increasing the intensity of such a stimulus, by increasing the speed of its movement and by repeating it on the same spot the sensation assumes a quality of itching. In his last review on cutaneous sensations von Frey (27) admitted that tickling might be a mixture of pure tactile impulses and itching. He also pointed out the great difference between a weak tactile sensation and tickling, the first being "objectivated", that is, related to external objects, and the second being "somatized", that is, related to the body or felt as an excitement of the body itself. It will be shown in this review that tickling and itching are identical. Electrophysiological analysis has revealed that both represent impulses of the

same nerve fibers and central tracts. Clinical experience conforms with this statement [see also (23, 26)].

*Experimental itch stimuli.* The first experiments on itching were carried out with "itch powder" (powdered filaments of the plant Mucuna pruriens, used by children in practical jokes). After a short period of pricking pain and a sensationless interval, it provokes pure itching in its full psychic content (57, 36). With itch powder a rather large surface of the skin was always stimulated, and it was assumed that a synchronous stimulation of numerous nerve endings is necessary to produce itching. In fact, it was found (25) that itching arises more easily from any suitable stimulus, especially from weak pain stimuli, if numerous neighboring skin points are stimulated. By such multiple stimulation a mutual accentuation of the impulses occurs. High frequency of repeated stimuli at the same place also furthers the inducement of itching. On this basis swinging tuning-forks (77) and faradic stimulation (16) were used to evoke itching experimentally. However, neither synchronous nor successive multiplicity of the stimulus is absolutely necessary to cause itching. It can be provoked by single, point-like, weak pain stimuli (4, 25). At present histamine punctures are preferred as experimental itch stimuli (7).

The whole body surface responds to itch stimuli. It is remarkable, however, that the highest pruritic sensitivity is found around the large openings of the surface in the transitional tissues between skin and mucous membranes (51).

Itching arises exclusively in the epidermis or in the corresponding epithelial layer of the transitional mucous membranes. It is impossible to elicit itching from skin areas denuded of their epidermis (71, 36).

*Physiologic pruritus.* Physiologic pruritus is a term used to contrast the pruritus of pathologic processes with the itching which arises in response to weak stimuli of every day life, such as slight rubbing, slight changes in pressure, and temperature (e.g., in undressing). The impulses resulting from such stimuli are of minimal intensity and scarcely enter the consciousness. However, a person may become conscious of this "minimal pruritus" when his attention is concentrated on it as occurs in forced immobility, in boredom and in fatigue (61). The instinctive desire of animals for cleanliness is supposed to be caused by it (35).

Itching evoked by pathologic processes in the skin or nervous system is merely an intensification of the physiologic pruritus and no sharp limit can be drawn between the two. However, such a distinction is

practicable if pathologic pruritus is interpreted as itching evoked by morbid stimuli or of such intensity that it causes disturbances of well-being. The most important feature of pathologic pruritus is the vicious circle of itching and scratching (see p. 376) which does not occur in physiologic pruritus although the latter is followed by short periods of scratching.

*Itching and pain.* Since the first experiments on itching were made, it has been obvious that the itching sensation is somehow related to cutaneous pain. It was found that in pathological cases of sensory dissociation itching could not be elicited in analgesic areas with intact touch sense, as in leprosy, syringomyelia and other lesions of the spinal cord (71, 4), and, conversely, itch sensation could be obtained in skin areas with complete tactile anesthesia and unimpaired pain sense, as in artificially induced sensory dissociation and in tabes (77, 57). Experiments on the normal mucous membranes showed the independence of itching from the temperature senses (57).

Special points on the skin surface which would respond with itching alone to any stimulus, such as is the case with touch, pain, warmth, and cold points, could not be detected but it was found that pain and itch points have the same density and that they cover each other (25). The interpretation of all these results was that itching is mediated by pain receptors and pain fibers. Some contradictory data concerning the relations of itching to tactile receptors apparently were based on too broad a definition of itching (16, 17, 40).

Further observations indicated that itching might be elicited in *hypoalgesia* by stimuli which normally caused pain. If the sensory functions of the skin were paralyzed by local anesthetics, locally or intraspinally, responses to pain and itch stimuli disappeared and reappeared at about the same time and independently of touch sensitivity. However, during the periods of increasing and decreasing anesthesia there were phases of hypoalgesia in which pain stimuli did not elicit pain, but itching (69). Similar observations could be made if the successive breakdown and recovery of skin sensations were provoked by blocking and releasing the blood circulation in limbs (49). In skin areas with *hypoalgesia* due to lesions of the spinal cord, slight pain and itching could hardly be differentiated. In such cases slight pin pricks were not felt at all; stronger pricks elicited an unpleasant sensation, called "itching pain" by the patient (57).

However, itching can be observed also in *hyperalgesic areas* and here elicited more easily than normally. Goldscheider (30) found that if

the skin is crushed in a small spot, a large area around the injury becomes *hyperalgesic* and in this zone itching can be elicited by subthreshold pain stimuli. Itching arises in such areas also by "inadequate" stimuli such as light point-like touch, cold and warmth.

The main difficulty in attributing itch sensation to impulses of pain fibers was seen in the fact that subjectively pain and itching are different sensations and call forth different motor responses. It seemed that an identification of pain and itching is incompatible with Muller's law of the specific energies of sense organs. This law, however, "has hitherto resisted all attacks, and all new facts in the physiology of sense organs have satisfied it" (79).

To overcome this difficulty, reference was made to the obvious unhomogeneity of the cutaneous pain sensation itself (pricking, stinging, burning, throbbing pain, etc.) and attempts were made to find a pain quality which in its psychic content is more closely related to itching than is the total complex of pain sensations; thus, the view was advanced that itching arises from impulses of the protopathic fibers of Head, itching being nothing else but a weak protopathic pain (57, 59).

*Criticism of Head's dualistic theory.* Head's theory (33, 34) was based on his findings in skin areas after the sensory nerves were cut and resutured. For a long time such an area responded only to crude pressure and crude thermal and pain stimuli, without spatial and gradual discrimination. Pain sensations were diffuse, poorly localized, highly unpleasant and with increased threshold. Fine tactile and temperature sensations, with sharp, point-like localization, and spatial and gradual discrimination did not reappear until several months after the nerve suture. On this basis the theory was advanced that there are two independent sensory fiber systems in the skin, the protopathic and the epicritic. The protopathic is the older and the less differentiated system. Acting alone it mediates explosive responses of an "all or nothing" type. The epicritic is the younger and more differentiated system. It serves to inhibit the abnormal intensity and faulty localization of the protopathic sensitivity.

Insufficient evidence is available for this theory and, in addition, factual discrepancies were found when Head's experiments were repeated (72, 63, 42). The strongest criticism was delivered by Lanier, Carney and Wilson (42), who repeated Head's experiments exactly and found facts which they felt differed in practically every point from Head's observations. Nonetheless, it is a conspicuous fact, and proves the inspirational value of Head's theory, that reference is still very

frequently made and consideration given to it in spite of the necessary reservations (2, 67), probably because, regardless of any theory, the existence of a protopathic sensitivity and its occurrence in a pure form is a clinical fact, familiar to every one who has to deal with sensory disturbances in lesions of the peripheral and central nervous systems. Even Lanier et al. under the heading of "Qualitative changes in sensitivity" describe "protopathic pain" in contrast with "normal pain" as an extremely uncomfortable and itching pain radiating over a wide area and tending to induce scratching. They also describe protopathic touch sensation. Finally they describe the hyperalgesic state in which incidental contact is felt as unpleasantly as in a bad sunburn. This state is very important in the understanding of the itch sensation. Of course, the term protopathic is used by these authors merely as descriptive and does not imply a special nervous mechanism. Their description, however, of the qualitative changes during nerve regeneration conforms to that of Head.

The main difference from Head's observations concerned the time relations in the return of protopathic and epicritic sensitivity. These time relations were such that in the interpretations of the authors, the theory of two independent fiber sets and the theory of suppression of protopathic sensations by epicritic sensitivity became untenable. In another part of this review, however, more recent experimental and clinical results will be reported which support both theories.

*Itching and protopathic pain.* The psychological analysis of the sensations, itching and protopathic pain, strongly suggested their close relation. Both protopathic pain and itching are characterized by poor localization, irradiation, poor discrimination of differential intensities, persistence of the sensation after the cessation of stimulation and by pronounced discomfort. On the contrary the epicritic pain sensation as elicited by sharp and short pin pricks is point-like, sharply localized and of short duration and has no similarity to the itch sensation.

Experimentally, when skin areas were tested whose epicritic sensory functions were lost in consequence of peripheral nerve lesions, distinction between itching and pain could not be made by the patient (57). With increasing intensity of the stimulus, itching went over into protopathic pain without any qualitative change of the character of sensation. Such a transition does not occur under normal conditions because the more intensive protopathic sensation is intermingled with, and more or less suppressed by, epicritic sensations. A weak pain which is

purely protopathic occurs more often in every day life and is identical with itching. In this conception scratching was interpreted as an expedient to suppress the very unpleasant protopathic impulses by the more tolerable epicritic pain.

*Double pain sensation.* In response to a single, short, pointlike, painful stimulus such as a pin prick a succession of two pain sensations can be observed. The two sensations are separated from each other by a sensationless time interval. The first pain sensation is short, pointlike, and can be sharply localized. The second is radiating, has an itching or burning character and is more unpleasant than the first (3, 49). In 1933 Zotterman (78) found that during the progressive loss of sensory functions of the skin induced by circulatory arrest of the limbs, there was a phase in which the reaction time of the pain sensation to a needle prick was increased to values of the same order as those found by Thunberg (70) for the second pain sensation. In other words, in local asphyxia a phase of sensory dissociation could be found in which the first pain was lost but not the second pain. The same type of sensory dissociation was studied in detail by Lewis and Pochin (49, 50), and they also found that under ischemia, loss of the first pain occurs much earlier than that of the second. Local anesthetics paralyze sensory functions in an order reverse to that of ischemia and hence, in cocaine anesthesia second pain is lost earlier than first pain. In circulatory arrest sensory qualities are lost in the following order: touch . . . cold and warmth . . . first pain . . . second pain. In local anesthesia second pain is lost first, and then first pain . . . cold and warmth . . . touch. These results could be related to findings of Clark, Hughes and Gasser (10) on the conduction rate of pain-mediating fibers. They found a close parallel in time relations between the successive disappearance of sensations in man during asphyxial nerve block and the successive disappearance of the electrical potential waves in electroneurograms of peripheral nerves in animals. It has been shown that the fibers of mixed peripheral nerves conduct afferent impulses at different conduction rates which are related to the size of the fibers. The A and B potential waves in the electroneurogram of peripheral nerves represent impulses of relatively large, fast-conducting fibers, whereas the late C waves are due to impulses in small fibers with a slow conduction rate. In human beings when, during asphyxial nerve block, sensations of pain and warmth alone can be evoked distal to such a block, in animals only C fibers remain active and their stimulation cause reflexes due to pain (10). It is true that there is a certain

overlapping of these pain responses towards the B waves, and it has been assumed that pain may be conveyed by both B and C fibers (28).

A much closer and clearer accord can be established, however, if C fibers are related not to pain generally but to second pain only because in ischemia C potential waves disappear at the same time that second pain disappears. Fibers of fast conduction rate and fibers conducting the first-pain impulses are more susceptible to local asphyxia. Fibers of slow conduction rate and fibers conducting second-pain impulses are more susceptible to cocaine (49). These results suggest that double pain sensations are due to impulses in two different sets of nerve fibers. The point-like, sharply localized first-pain sensation is conducted by fibers of fast conduction rate (B fibers); the second-pain sensation which has a burning, itching, radiating character follows later, because it is mediated by the small C fibers of slow conduction rate.

Definite evidence of two separate pain fiber sets was presented by Lewis and Pochin (49) when they showed that the time intervals between first and second pain progressively diminish with shortening of the pathway from the spot of stimulation to the central nervous system. Because the peripheral pathway is longest from the toes and fingers respectively, the time interval is longest if the pain stimulus is applied at these points. When one approaches the hip and shoulder, the peripheral pathway becomes shortened and the time interval diminishes steadily, until the difference in conductivity rate of the two pain conducting sets is not large enough to make two distinct sensations, and the first and second pain become entirely fused.

Pochin (54) also presented evidence that the delay of pain perception in tabes is due not to an abnormal slowing of impulses but to a defect in that group of pain fibers which conduct rapidly, and reveals the effects of a second group of fibers which conduct slowly (cf. 44).

*Itching and tickling.* It was mentioned on page 357 that earlier investigators were inclined to attribute "superficial tickling" to impulses arising in tactile receptors. The existence of specific tickle receptors has also been assumed (4). However, more recent clinical experiments showed that tickling sensation is intimately connected with the pain-mediating fiber system.

Pritchard (55), first stating that superficial tickle elicited by drawing a hair across the skin, is always highly unpleasant and leads to the motor response of rubbing or scratching, practically identified tickling with itching. In patients suffering from itching skin lesions he found

that itching attacks started with a tickling sensation which went over into itching without critical qualitative change of the sensation. Skin areas surrounding itching spots did not show hyperesthesia to touch but did show hypersensitivity to tickling. The threshold pressure necessary for tickling was not lowered, but, on equal stimulation, tickling was elicited more easily and more intensely around itching spots than in normal skin.

In patients suffering from peripheral neuritis, hypoesthesia to touch was combined with exaggerated responses to pain and tickle stimuli. Even when hypoalgesia was present, with its raised pain and tickle threshold, once the threshold was reached, the pain and tickle were unpleasant, almost to the point of being intolerable.

This hypoalgesia, in which tickle, itching (57) and pain responses are exaggerated, fits Head's description of protopathic sensitivity, with its raised thresholds and explosive sensory perceptions. On this basis tickle has to be regarded as a part of protopathic sensitivity.

Further close relations of tickling to pain have been revealed by Pritchard in his examinations of sensory disturbances in patients with lesions of the central nervous system. In all lesions of the spinal cord, thalamus and brain, pain and tickling were altered in parallel whether they were exaggerated or normal, weakened or totally absent. Thus, clinically, hyperalgesia may be detected by exaggerated tickling sensitivity, and absence of this sensitivity means impairment of pain conduction. Identical results were obtained by Foerster (21) in his neurosurgical experiments. Section of the anterolateral tract of the spinal cord abolishes tickling and itching. Such an operation at the corresponding level stops the most severe pruritus ani or pruritus vulvae immediately. In the corresponding area the patient feels normally and differentiates dull and sharp sensations but even the sharpest one does not cause any unpleasantness, and thus it cannot be called pain.

On the other hand, section of the posterior tracts in the acute experiment produces "hyperpathia," exaggerated tickle, itching and protopathic pain responses. "Posterior and anterolateral tracts serve both touch and pain, but the latter mediates only a primitive, non-differentiated touch and pain sensation. The accessory impulses by which the differentiation of kind and origin of the tactile or pain stimulus is recognized, is due to impulses in the posterior tract" (21). These impulses exert a suppressing influence on the impulses of the anterolateral tract. The suppression occurs centrally in the thalamus and the cortex.

Recently on the basis of clinical experiments it was stated again that itching, if it is evoked by mechanical stimuli, always has a tickling component (37). The quoted results do not exclude the participation of tactile receptors in tickling impulses. Pritchard (55) considers the possibility that pressure receptors may initiate impulses which provide excitation of the pain pathway in the cord, if the frequency of repeated stimulation is sufficiently high. However, he points out that this supposition is in contradiction to Adrian's statement (2) that even the most frequent touch stimuli do not cause pain. It seems therefore more probable that the "tactile component" of the tickling impulse is set up in those pressure receptors which mediate primitive, non-differentiated touch sensation, which have a low rate of adaptation, and which tend to summation and to produce after sensations. It will be shown in the next paragraph that the primary (protopathic) touch sensation of tickle has its counterpart in the electroneurogram of the impulse.

*Electrophysiological analysis of itching* (79, 80). All conclusions quoted in the foregoing paragraphs were more or less based on introspective observations of patients and normal test persons and might have been subject to error. However, application of the more recent methods of electrical registration of afferent nerve impulses as developed by the work of Adrian (2), Erlanger and Gasser (18), Bishop (9) and others, led to the same conclusions as did the clinical work, namely, that tickle, itch and protopathic pain are functions of the same set of nerve fibers. Such evidence was produced in animal experiments hy analysis of the electroneurograms of impulses caused by different cutaneous stimuli which in their turn were analyzed for sensations they might cause in man.

In the experiments of Zotterman (79, 80), the impulses after the delivering of touch stimuli, such as moving of the hairs or painless deformation of the skin, showed in their electroneurograms large potential waves (A and B) which were due to impulses in fibers of more than 10 $\mu$ in diameter and of a conductivity rate of 30 to 60 meter/sec.

If pure noxious stimuli were applied without deformation of the skin, such as radiant heat or chemicals, the recorded amplitudes of the axon potentials were very low. They corresponded with the C waves of Erlanger and Gasser (18), i.e., with impulses of very small non-myelinated fibers with diameters below 5.5 $\mu$ and a conduction rate of 0.7 to 1.3 meter/sec.

If touch or deformation was combined with pain stimuli, as in the case of pinching or pricking, there was a sudden response of large fibers

to the deformation and then a response of the very small fibers, which continued to respond in after-discharges with decreasing frequency.

Beyond that, any stimulus which elicited pricking pain was followed by "$\delta_1$" potential waves representing impulses of fibers of less than 10 $\mu$ in diameter and of a conduction velocity of 20 to 30 meter/sec. These fibers are the largest in the spinothalamic tract and probably are myelinated. One found waves not only in responses to pin pricks but in responses to firm strokes as well. Thus, it seemed that the sharp "first pain" was due to impulses of specific fibers which always produced the same sensation independent of the kind of stimulus. On the contrary, the delayed "second pain," characterized by radiation, itching or burning quality and persistence after the stimulus had ceased, was due to slower impulses of non-myelinated fibers and appeared after any kind of burning stimulus and as an after-effect of needle pricks.

In tickle provoked by a light stroke with a piece of cotton, "$\delta_2$" potential waves were recorded representing impulses of fibers of 5.5 to 7 $\mu$ in diameter and of a conduction rate of 8 to 17 meter/sec. Weak after-discharges in the C fibers corresponded with the itching after-sensation caused by such a stimulus.

The after-sensations following light strokes, firm strokes and burning stimuli on the skin could not be qualitatively differentiated. Itching and burning sensations fused as the strength of the stimulus was increased. In the electroneurogram all these impulses were represented by C potential waves. Their frequency increased with increasing intensity of the stimulus but their quality did not change. This means that *an increase in the stimulation of C fibers does not alter the character of the sensation when tickling progresses to itching and then to burning. The after-sensations of tickling and burning pain are identical with itching and are mediated by impulses of C fibers.*

Zotterman's work confirmed the law of specific energies distinguishing the potential waves belonging to fibers which mediate touch, on the one hand, and different kinds of pain, on the other. In 1935 Adrian (2) still considered the hypothesis of Goldscheider (29, 31) that both pain and touch sensations might be evoked by impulses in the same fibers, the difference in reaction depending only on the intensity of stimulation. Although it was clear at that time that the nervous apparatus responding to light touch did not mediate pain sensations and although evidence was presented that C fibers carried protopathic pain impulses, it was not clear that they conducted pain only. Thus the existence of specific fibers seemed to be doubtful.

In Zotterman's analysis the C fibers appear as carriers of uniform afferent impulses always causing the same quality of sensation independent of the kind of stimulation. Light and firm strokes as well as noxious pain stimuli have qualitatively the same late effect. Tickling, itching and protopathic pain are felt according to the frequency of impulses in the same fibers.

An objective basis for the poor localization of second pain and itching was found by Zotterman in polyphasic configurations of the corresponding axon potentials indicating the frequent branching of the axon.

Finally, the electroneurograms of Zotterman demonstrated the suppressive action of the fast epicritic impulses, travelling up the posterior columns, on the protopathic impulses which travel slowly in the anterolateral tract and arrive later in the center. This finding must be regarded as strong supporting evidence for the conception of Head.

*Hyperalgesia* (44, 45) *and itchy skin* (7). In 1916 Goldscheider (30) found that after crushing a circumscribed spot of the skin, a widespread area of surrounding hyperalgesia developed. In this area itching often was felt very unpleasantly, particularly after slight touching or stroking, and, as an after-sensation, to cold stimuli in this area.

The mechanism of this phenomenon was thoroughly investigated and clarified by Lewis in 1936 (44, 45, 46). He found that this hyperalgesia might be provoked: 1, by any painful injury of the skin; 2, by stimulation of a cutaneous nerve trunk, and 3, by stimulation of a small branch of a cutaneous nerve. In all three instances the hyperalgesia spread within or actually filled the cutaneous nerve territory in which the stimulus fell. The hyperalgesic area was more or less oval according to the shape and size of this territory. Its long axis was parallel to the nerve trunk and might be as long as 5 to 20 cm. The hyperalgesia was fully developed in 10 to 20 minutes and might last many hours. Needle pricks in this area caused unusually intense, diffuse and long lasting pain, whereas touch stimuli elicited soreness. The response to warmth was slightly increased.

This hyperalgesic state was not caused primarily by the effect of painful stimuli on the central nervous system. In other words, it was not referred from the brain or spinal cord. If block anesthesia was made previous to the crush so that the crush was unfelt, the usual area of hyperalgesia developed around the injury as soon as the nerve recovered from the block. The observation that the hyperalgesia closely filled the territory of the corresponding cutaneous nerve irrespective of the place and type of stimulation strongly suggested that the hyperalgesic state was established by a local nervous mechanism.

This was definitely proved by experiments with intradermal injections of local anesthetics. If a tiny crush was made in a small procaine wheal, hyperalgesia did not develop around the crush as long as the anesthesia persisted but developed in the usual way as soon as the skin recovered from anesthesia. Development and involution were simply postponed. Thus, hyperalgesia was not due to the spread of a pain producing substance into the neighboring skin for the diffusion of such a substance would not be hampered by block of the small nerve fibers which were hit by the local anesthesia. However, a persistent change was produced by the injury. The capacity to provoke hyperalgesia resided in and was maintained by the crushed skin; the effect of injury (chemical products?) acted through local nervous channels as soon as these became unblocked.

The action, as described by Lewis, occurred through local nervous channels and, being independent of the central nervous system, was explicable only on the basis of what has been called an axon reflex, the impulse traveling up one branch of an axon and returning from the point of ramification through side branches. Since the hyperalgesia originating in a small area might spread in all dircctions to include a much larger territory, it had to be assumed that a system of nerve fibers connected the small territory to almost every part of the larger hyperalgesic territory within the area of a given cutaneous nerve trunk. This system might be a network or a complex of overlapping arborizations of axons. The latter type was suggested on the basis of experiments with small novocaine barriers within the area of spreading. If the injury was made eccentrically within an intradermal novocaine wheal or outside but close to it, hyperalgesia developed to one side but failed to pass beyond the barrier until the effect of local anesthesia had worn off. The spread was blocked as soon as the way of the impulse from final arborizations to the parent axon was blocked. It did not creep around the barrier, as it would do if the impulse spread through a continuous network. Such a creeping, however, was observed when the injury was made far enough from the barrier so that the impulse might reach the point of ramification in the parent axon; then the impulse might return in every direction through side branches of the widely ramified axon. This type of impulse in widespread peripheral arborizations of a nerve trunk is identical with the type which must be assumed in order to explain the widespread pilomotor and sweat response to faradic stimulation and to intradermal injec-

tions of acetylcholine and other drugs with nicotine-like action (48, 8, 75, 13, 60).

Lewis (44) has shown that the hyperalgesic state as described above is not a function of sympathetic fibers. It occurs unchanged in areas deprived of sympathetic innervation. Lewis also dismisses the rôle of sensory nerves in the development of the hyperalgesic state for two reasons: first, he regards pain as an accurately located sensation and states that such a sensation cannot be elicited in a system of branching axons. Secondly, he finds that in sensory dissociation by circulatory arrest and by local anesthetics the disappearance of pain perception and of the hyperalgesic state are separate. "Cutaneous pain nerves merely register, through sensations called hyperalgesia, a state of the skin for which they themselves are in no way responsible." Lewis assumes the existence in the skin of a hitherto unknown nervous system and discusses the possibility of its identity with nerve plexuses involved in antidromic vasodilatation and in the state of erythralgia (43). Particularly emphasized is the similar behavior of the "flare" and the hyperalgesia, both spreading around local skin injuries in a similar distribution. The nervous system in question is associated with local defense against injury by protective hyperalgesia and by protective hyperemia and is therefore appropriately called the "nocifensor" system.

To exclude the participation of pain fibers in the mechanism which leads to hyperalgesia for the reason that pain sensations are sharply localized, whereas the hyperalgesia is based on a special state of widely ramifying axons, seems to be correct concerning epicritic pain but can hardly be accepted in regard to the protopathic quality. On the contrary, according to the description the hyperalgesia around the point of painful stimulation is identical with a state which might be called "protopathic hyperalgesia" or "hyperpathia" (21) and which is well known and described in lesions of the peripheral and central nervous system as well as in itching skin lesions, particularly in gross lesions of the epidermis ("eczematiform" reactions). Lewis' second reason for ruling out sensory nerve fibers is not easy to eliminate. He finds that, whereas the second pain sensation is the first sensory perception to be lost in cocaine anesthesia and the last one to be lost in asphyxia, the capacity for developing a hyperalgesic state shows an almost opposite behavior, namely, its very early loss in asphyxia and its late loss in cocaine anesthesia. What is more, the experiments of Lewis prove

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tions of acetylcholine and other drugs with nicotine-like action (48, 8, 75, 13, 60).

Lewis (44) has shown that the hyperalgesic state as described above is not a function of sympathetic fibers. It occurs unchanged in areas deprived of sympathetic innervation. Lewis also dismisses the rôle of sensory nerves in the development of the hyperalgesic state for two reasons: first, he regards pain as an accurately located sensation and states that such a sensation cannot be elicited in a system of branching axons. Secondly, he finds that in sensory dissociation by circulatory arrest and by local anesthetics the disappearance of pain perception and of the hyperalgesic state are separate. "Cutaneous pain nerves merely register, through sensations called hyperalgesia, a state of the skin for which they themselves are in no way responsible." Lewis assumes the existence in the skin of a hitherto unknown nervous system and discusses the possibility of its identity with nerve plexuses involved in antidromic vasodilatation and in the state of erythralgia (43). Particularly emphasized is the similar behavior of the "flare" and the hyperalgesia, both spreading around local skin injuries in a similar distribution. The nervous system in question is associated with local defense against injury by protective hyperalgesia and by protective hyperemia and is therefore appropriately called the "nocifensor" system.

To exclude the participation of pain fibers in the mechanism which leads to hyperalgesia for the reason that pain sensations are sharply localized, whereas the hyperalgesia is based on a special state of widely ramifying axons, seems to be correct concerning epicritic pain but can hardly be accepted in regard to the protopathic quality. On the contrary, according to the description the hyperalgesia around the point of painful stimulation is identical with a state which might be called "protopathic hyperalgesia" or "hyperpathia" (21) and which is well known and described in lesions of the peripheral and central nervous system as well as in itching skin lesions, particularly in gross lesions of the epidermis ("eczematiform" reactions). Lewis' second reason for ruling out sensory nerve fibers is not easy to eliminate. He finds that, whereas the second pain sensation is the first sensory perception to be lost in cocaine anesthesia and the last one to be lost in asphyxia, the capacity for developing a hyperalgesic state shows an almost opposite behavior, namely, its very early loss in asphyxia and its late loss in cocaine anesthesia. What is more, the experiments of Lewis prove

definitely that asphyxia does not interfere in any way with the mechanism that produces the hyperalgesia but acts directly on the "nocifensor" nerves themselves. It must be admitted that this peculiar behavior can hardly be explained otherwise than by the existence of a system independent of pain perception.

Even if in the future a simpler explanation were found for the different behavior of protopathic pain and hyperalgesia in circulatory arrest and in local anesthesia, and even if hyperalgesia were recognized as a special hyperactive state of the slow conducting C fibers, as believed by the reviewer, it still would remain an important discovery that the nervous apparatus in question forms a branching and rebranching system of axons within the territory of a cutaneous nerve. By this statement, as previously mentioned, a remarkable similarity with the sympathetic fibers of the skin has been established and it may be pointed out that this similarity seems to concern *two phylogenetically older and less differentiated systems of small and unmyelinated fibers, which mediate diffuse effects, in contrast to the newer and highly differentiated epicritic sensory system.* The capacity of sharp localization in the latter is evidently incompatible with ramifications of the axons. The type of diffuse and exaggerated sensory responses as they are frequently found in pathologic conditions of the nervous system, skin and mucous membranes is more clearly understood by an acknowledgment of the findings of Lewis.

The close relation of these findings to the problems of itching became evident by Bickford's observations on "itchy skin." Bickford (7) found that after applying a stimulus which causes itching, two different phenomena occurred: 1, there was a local itching confined to the point of stimulation which persisted, after the stimulation had ceased, without the intervention of further stimuli and was therefore called "spontaneous itching"; 2, around the point of stimulation a widespread area could be mapped out which did not itch spontaneously but responded with itching to light friction. This area was called "itchy skin." In complete accord with the hyperalgesic area of Lewis the area of itchy skin was oval shaped with its long axis parallel to the corresponding cutaneous nerve trunk and it filled closely the territory of the nerve. Evidence has been presented that it spreads through axon ramifications as the hyperalgesia does.

The close relation between hyperalgesia and itchy skin is indicated by their alternating appearance in the same individual after more or less painful stimulation. There are also individual differences in the

response to identical stimuli, some persons signaling hyperalgesia, others, itchy skin. Furthermore the hyperalgesic area might be surrounded by a narrow zone of itchy skin (44, 7).

"Spontaneous itch" and "itchy skin" could be separated in sensory dissociation caused by local asphyxia and by local anesthetics exactly in the same way as pain and hyperalgesia.

"Itchy skin" playing a prominent rôle in skin diseases has been known and described long ago under the heading of "increased itching sensitivity" (58, 59). It has been stated that "itching skin lesions," such as urticarial wheals, prurigo nodules, eczematiform vesicles and lichenified lesions, itch "spontaneously" only during their development while irritating substances are being formed locally by the morbid process. These irritating substances are responsible causally for two independent phenomena: 1, the anatomic (inflammatory) lesion itself, provoked by cell injury, and 2, the itching, provoked by stimulation of the nerve endings. This itching is of short duration, usually lasting only a few minutes. However, after this itching has subsided an increased itching sensitivity persists for many hours or even days, in the area of the lesions, regardless of whether the anatomic lesion has subsided, as in urticaria, or has not, as in the case of eczema.

Even if the increased sensitivity is not very pronounced, as in urticaria, itching may always be elicited by rubbing, hours or days after the urticaria has subsided. This phenomenon is a part of what has been called somewhat poetically "mnemodermia" or cutaneous memory (35, 68). There is no itching skin disease in which it cannot be found.

If the persisting excitability is high, the threshold for adequate stimuli is considerably lowered and the responses are exaggerated. In addition, inadequate stimuli, such as light touch, light strokes, pressure, release of pressure and temperature stimuli, elicit violent itch sensations. In such spots, one might say, even the slightest breath of air may evoke itching, and, under the conditions of every-day life, it becomes practically impossible to avoid all the trivial stimuli causing the itch sensation. Although in reality this condition is a state of "itchy skin," or itching hyperexcitability, it is mistaken for a state of "spontaneous itch" because the slight and inadequate, but very effective, external stimuli escape notice (58, 59).

The responses of itchy skin areas to different stimuli were as follows: 1, needle prick revealed definite hyperalgesia, intensification, prolongation and radiation of the pain sensation, and itching after-sensation; 2, itch powder caused greatly intensified, radiating and prolonged itching;

As the triple response is always accompanied by itching the question has arisen whether this sensation, too, may not be provoked by the release of H-substance. The idea was that possibly any physical or chemical stimulus which provokes both the triple response and itching, does not act directly on cells and nerve endings but through the mediation of the released H-substance. The supporting evidence for the theory that itching is effected through chemical mediation was presented by Lewis, Grant and Marvin (47) who showed that if the circulation is arrested in one arm and then both arms are uniformly stimulated the itching is intensified and greatly prolonged on the arm with arrested circulation. The period of itching may be prolonged by the whole time duration of circulatory arrest. The interpretation of this observation was that "the released substance causing itching is held in situ and continues to act on nerve endings during circulatory arrest" (43).

The conception of a uniform chemical mediation of the triple response and of itching had a fertile influence on the interpretation of pathologic processes in the skin, particularly when the observations were extended to the responses in anaphylactic and allergic skin reactions (43). These reactions and the responses to histamine showed an identical behavior under various conditions and it has thus been assumed that the anaphylactic or allergic antigen-antibody reaction in the skin leads primarily to the liberation of H-substance in the same way as injury does. This conception has been of great importance in respect to itching because a great number of itching skin lesions are based on hypersensitivity reactions (59). The two prototypes of such lesions are: 1, the urticaria, in which the site of the hypothetical sessile antibodies is the cutaneous vessel wall (urticaria is a "local anaphylactic shock," 15), and 2, the eczematiform vesicles, in which the site of hypersensitivity is supposed to be in the epidermal cells. A further fact in favor of the histamine theory of itching in anaphylactic reactions is that pruritus may be the first or even the only symptom of the systemic anaphylactic shock in animal experiments as well as in human beings (39). This itching might be related to increased histamine content in the circulating blood during the anaphylactic shock (19).

It seems compatible with all known facts to assume that in itching cutaneous reactions based on hypersensitivity, itching is mediated by the liberation of histamine or a similar substance. According to this view anaphylactic or allergic skin reactions and itching are coördinated phenomena, both caused by the same chemical agent. However it must be emphasized that all evidence for this view, though impressive, has been indirect. Attempts to isolate histamine or a similar substance

from these lesions have not been successful. For this reason the term "H-substance" was chosen.

Direct evidence for liberation of histamine in excised pieces of skin when they are subjected to painful electrical stimulation has been presented recently (56). The experiments strongly support the theory that liberated histamine may be a chemical mediator for cutaneous pain.

In this whole problem one thing is certain, namely, that neither itching nor cutaneous pain is mediated in every instance by histamine or a similar substance. This conclusion can be drawn from the fact that histamine in as high a dilution as 1:600,000 to 1:1,000,000 still causes whealing (43); this dilution, however, does not cause either pain or itching in a higher degree than physiological saline. It appears that histamine can be responsible for cutaneous pain or itching only in those instances in which they are accompanied by whealing or other inflammatory changes according to the site of liberation.

Kenedy (37) has discussed the most likely possibility, that itching may be mediated by histamine in certain cases but not in others. Many conditions are known to cause violent itching without any gross or microscopic changes of the skin; such as the pruritus in pregnancy, diabetes, diseases of the liver and of the hematopoietic system. Whether in these cases the metabolic products of the pathologic process act directly on the nerve endings or by intervention of a chemical mediator, is not known but it can be stated that histamine cannot be the mediator because nothing is seen on the skin. In concentrations which evoke itching, histamine causes visible changes. "Pruritus sine materia" (pathologic itching without visible changes) is not mediated by histamine.

*The scratch reflex.* The motor response to itching is a spinal reflex and can be elicited in the spinal dog from a saddle-shaped area of the back by mechanical or electrical stimulation (64, 14), and in the cat by mechanical stimulation of the external ear (11). The scratch movements are carried out with the homolateral hind limb. The rhythm of the movement is based on refractory periods developed in spinal centers and is independent of the frequency of the stimulus (64). The reflex is disynaptic and is brought about through the anterolateral and anterior tracts of the spinal cord (64). After bilateral ablation of the frontal cortex the scratch reflex becomes considerably enhanced (11). Thus the existence of an inhibitory mechanism of higher centers on the scratch reflex has been established.

Itching and scratching can be provoked in cats also by partial sensory

denervation producing a state of "itchy skin" or protopathic hypersensitivity. In this condition scratching is so violent that large pieces of skin are torn off and deep excoriations are made which heal with scars (5).

In human beings the different forms of the motor response to itching, such as scratching with the nails, rubbing or kneading the skin, are controlled to a certain degree by consciousness and will power similarly to the defense reflex to pain stimuli. Itching was interpreted as a "foreign body sensation," and scratching, teleologically, as a suitable defense reflex for removal of noxious foreign bodies (53).

There are two instances in which pathologic itching may be stopped by scratching: first, when itching is actually caused by foreign bodies which can be removed from the skin by scratching, and second, when, by scratching, the epidermis, in its entire thickness (and with it the epidermal nerve fiber nets) is torn off. This happens in cutaneous diseases either by tearing off elevated lesions (e.g., prurigo papules) or by tearing off the flat skin surface with "digging" movements of the nails (e.g., neurotic excoriations). In these cases itching ceases immediately in loco.

In all other instances scratching is unsuited for stopping itching. As long as one scratches, there is relief because the more tolerable epicritic pain suppresses the intolerable itching. However, when the scratching is discontinued the itching starts again as an after-sensation following scratching or rubbing stimuli because these stimuli send impulses not only through epicritic but also through protopathic fibers. In pathological cases of "itchy skin" in which the protopathic sensitivity is over-accentuated, this situation leads to a tormenting vicious circle, consisting of increasingly violent scratching and increasingly intensive itching. Such scratch paroxysms may last many hours and may cease only because of total somatic and psychic exhaustion of the patient.[1] In chronic cases a further disturbing factor must be taken in account, namely, a special proliferative reaction of the epidermis to chronic mechanical stimulation called "lichenification," which exaggerates the hyperexcitability of the protopathic system. Whether this state is connected with new formation of nerve plexuses in the epidermis is not known but seems to be possible.

*Itching of central origin.* Centrally induced itching was observed

[1] Discussion of the alleged relation of scratching to sexual instincts (scratching a "cutaneous onanism" (35), "substitutive for sexual orgasm" (65)) are beyond the scope of this review. For details see (62, 66).

after intracisternal injection in the cat of 0.2 to 0.5 mgm. of morphine (52, 41, 76). Violent scratching, mainly of the ears but sometimes of the nose and other parts of the head and neck, by one of the hind limbs started a few minutes after the injection and lasted for from 1 to 1½ hours. After the attack subsided, a new attack of scratching could be provoked by stroking the ear. After local anesthesia of the ear or after section of its sensory nerves this peripheral inducement to scratching failed, but the effect of renewed intracisternal injection of morphine was not impaired. It was thus shown that the effect of morphine is directly on the center. Intralumbar, intravertebral and paravertebral injections of morphine were without effect.

Successive ablation of cortex, thalamus, corpus striatum and pallidum, midbrain, cerebellum and medulla to the level of the acoustic nucleus, did not influence the scratch response. If the medulla was sectioned at the level of the restiform bodies and the injection was made into the opened cerebrospinal canal rhythmic movements of the hind limb were still observed but the limb did not reach the head. After sectioning of the medulla at the level of the pyramid decussation real scratch movements were no longer seen, but some uncertain movements occurred. It was concluded that the main site of the scratch center ("Kratzwerk") is situated in the medulla below the acoustic nucleus.

In human beings itching evoked without peripheral impulses has been observed in diseases of the central nervous system, such as tabes, dementia paralytica, and more rarely in manic-depressive psychosis, idiocy and epilepsy.

The excitability of the central nervous system also influences the intensity of any kind of peripherally induced itching. If the itching is not very intense the attention can be diverted from it (77). On the other hand merely imagining the presence of biting insects may provoke itching and scratching in centrally hyperperceptive individuals.[2] Pharmacologically, caffeine intensifies the central perceptibility of itching (59), whereas bromides and the narcotics acting on the brain stem (phenobarbital and other barbiturates) have a beneficially depressing effect (17, 40).

It is an unexplained fact that morphine and the related derivatives of opium, the most effective drugs for central relief of pain, do not decrease the itching sensitivity but, on the contrary, usually increase it. Even in morphine-induced deep sleep scratch movements can be

[2] The relation of itching to functional skin diseases has been discussed in detail by Becker and Obermayer (6).

observed in patients suffering from itching skin diseases. Probably this itching is partly due to a peripherally stimulating effect of morphine, as morphine given intracutaneously elicits the triple response of Lewis with itching. Another possibility is that morphine may provide a state of cutaneous hypoalgesia in which pain stimuli tend to cause itching sensations (see p. 359). However a central stimulating effect on itching must also be taken in account on the basis of animal experiments quoted above.

As previously mentioned, local injection of epinephrine inhibits responses to itch stimuli. However, if itching has once been aroused in normal skin, the subsequent local administration of epinephrine does not inhibit further itching (10). This observation demonstrates the central summation of itching which may be compared with the central summation of pain (1).

*Therapeutic considerations.* If the causal factors in the development of an itching skin disease cannot be determined, the physician is compelled to treat itching as one of the predominant, troublesome and, because of the scratch reflex, intractable symptoms.

According to what has been said about "itchy skin" or itching hypersensitivity, there are, besides the necessity of decreasing central sensitivity, two principles governing such treatment: 1, reducing the visible lesions which are the basis of itching hypersensitiveness; 2, eliminating external stimuli as far as possible by use of suitable non-irritating clothing; by avoiding gross temperature changes; controlling sudden changes in pressure as they occur in undressing, in removing bandages, and in entering and leaving the bath. The understanding of itching hypersensitivity as a state in which inadequate stimuli elicit itching is of great importance in therapy (59).

## SUMMARY

Itching represents a sensory quality which is identical with that of protopathic pain. Superficial tickling progresses to itching and itching to diffuse burning pain without critical change of the quality of sensation when the intensity of the stimulus is gradually increased. They have a longer latent period than other cutaneous sensations because they are mediated by the slowly conducting C fibers of Gasser and Erlanger. Whether tickling, itching or protopathic pain sensation is felt, depends on the frequency of impulses in these fibers. The sensations persist after the cessation of stimulation, the fibers being inclined to after-discharges and their impulses being capable of central summa-

tion. They often appear as after-sensations of other sensory perceptions. The clearest example of such combined impulses in different fiber sets is the double pain sensation following needle pricks.

Itching is experienced in a rather pure form, and becomes accentuated or explosive if the epicritic sensitivity is depressed following injuries to the skin, to the peripheral nerve trunk, or to the central nervous system. Itching caused by pathologic processes is always based on such a condition. If the over-accentuation of protopathic sensitivity is pronounced, the slightest external stimuli, even if they are inadequate (e.g., touch and temperature stimuli) evoke itching.

Polyphasic configurations in the electroneurogram of "second pain" impulses indicate the frequent branching of the corresponding axons. The ramifying axon system revealed by Lewis in experiments on hyperalgesia, although interpreted differently, also seems to be related to the perception of protopathic pain and itching. Radiation and poor localization of these sensations are explained by their originating from richly ramifying axons. According to this view there are three ramifying axon systems in the skin, the sympathetic, the antidromic vasodilator and the protopathic sensory system, all consisting of small unmyelinated fibers and all mediating diffuse effects by means of axon reflex impulses. Sympathetic and protopathic sensory systems often respond in coupled reactions to cutaneous stimuli but itching arises without any interference of the sympathetic fibers. Itching and antidromic vasodilatation are also frequently coupled with each other but there is no evidence that vasodilatation is a necessary factor in evoking itching.

Supporting evidence has been presented for the theory that itching following skin injury and itching in skin lesions based on hypersensitivity reactions might be mediated by liberation of H-substance. However, liberation of histamine or of a similar substance cannot be responsible for itching which occurs without visible changes in the skin.

The central inducement of itching, the scratch reflex and therapeutic considerations are briefly discussed.

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# ENDOCRINES IN INVERTEBRATES

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The study of invertebrate endocrinology, although it provides interesting parallels to the hormonal activities in vertebrates, should be viewed not merely as an extension of endocrinological research to the invertebrates, but as a field presenting new questions of general importance. Among these is the problem of non-autonomous development of hereditary characters under the influence of diffusible substances ("gene hormones"), or the physiological significance of neurosecretion. It is with these and similar contributions which the study of invertebrates has made to endocrinology that this article is primarily concerned.

SEX HORMONES. Long before other hormonal activities became known in invertebrates the presence of sex hormones was discussed with respect to worms as well as molluscs and arthropods. However, there still is considerable controversy concerning the interpretation of the data available at present.

Evidence of the occurrence or non-occurrence of sex hormones in invertebrates has been furnished in different ways: 1, by the analysis of "parasitic castration," a phenomenon consisting mainly in a partial or total destruction of the gonads by parasites; 2, by experimental castration accomplished surgically or by irradiation, for from changes in secondary sex characters occurring after castration the presence of sex hormones is indirectly concluded; 3, by transplantation of gonads between the two sexes; 4, by regeneration experiments; 5, by assaying invertebrate material in vertebrates and vice versa (p. 403).

Among the *worms* the male gonads were found to control the development of the copulatory organs in turbellarians, for example, Planaria. Regeneration of these secondary sex characters failed in the absence of

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the anterior body region which contains the gonads, and in the case of parasitic castration (179). Similarly the differentiation of a secondary sex character typical for the annelids, the clitellum, seems to take place under the control of a male sex hormone. According to Hess and Bacon (202) this hormone originates in the seminal vesicles and not in the testes. For a discussion of other experimental data on this problem see Heumann (84).

Further evidence of an endocrine function of the gonads was obtained in *molluscs*. The hectocotylus, a secondary sex character of the cephalopods, depends in its development upon the presence of the gonads (163) and so do the accessory characters of the genital apparatus in the snail Littorina (115).

That sex hormones play a certain rôle in *crustaceans* appears now also probable, although only indirect evidence is available. Numerous cases of parasitic castration have been carefully analysed since the first observation of the phenomenon (64). In the crab Inachus, for instance, the parasites (Sacculina, fam. Rhizocephala) cause a varying degree of destruction of the gonads and consequently influence the secondary sex characters which show an outright dimorphism in the male and female hosts. Already existing characters disappear and are replaced to a certain extent by those characteristic of the opposite sex; i.e., the infected male becomes typically female in appearance. It is of importance that the alteration of secondary sex characters is not merely a return to the juvenile type, but an approach to the adult features of the opposite sex (177). In the majority of cases these changes toward the opposite sex concern the male, whereas the infected female in most cases loses only some of its own secondary characters. There are, however, a few indications that the female may assume a few characters more or less male (165). Although in all these observations the extent of the infection and the effects of it may vary considerably in regard to different hosts and various parasites (for a discussion of the extensive literature see 59, 102, 80), castration generally results in an alteration of the secondary sex characters. Interpretations of the findings are controversial (166, 67, 122, 178, 27, 102, 80); however, in the crustaceans an endocrine significance of the gonads seems undeniable. This view is supported by experiments in which sterilization of females by radium irradiation prevented the development of certain secondary sex characters such as the brood pouch in the isopod Asellus (74) or part of the cyclical secondary characters in the amphipod Gammarus (114). Similar conclusions as to the rôle of sex hormones in crustaceans were reached by various workers (10, 127, 128, 50, 56, 43).

Unlike the crustaceans, the *insects* responded with negative results to experimental castration or transplantation of gonads. After grafting gonads of the opposite sex into castrated caterpillars, Meisenheimer (121) observed no influence on the secondary sex characters of the adult moths. After surgical castration of larvae the respective external secondary sex characters developed fully in the resulting insects and the sexual behavior appeared to be unaltered, as described by a number of investigators (see 80).[2] In contrast to all these negative results, similar experiments in the Lepidopteran Cosmotriche (148), for instance, suggest a possible hormonal influence of the gonads on some, at least, of the secondary sex characters of this species (see also 53). Furthermore, an effect of this kind is clearly observed after parasitic castration. "Stylopization," i.e., castration by the parasite Stylops, first described in the bee, Andrena, by Pérez (see 189), results in changes of the secondary sex characters of both sexes. In infected males the color of the face (clypeus normally yellow) and the structure of the hind legs assume a female appearance. Similarly in a stylopized female, besides other changes, the pollen-collecting apparatus of the legs becomes almost as reduced as in the male and the color of the clypeus changes from its normal black to yellow. Most of the additional contributions to this problem are in accordance with Pérez' classical observations (see 102, 80). Thus recently again Rempel (151) found in Chironomus, parasitized by nematodes, the external genitalia "distinctly under the control of the sex cells, this control, as in vertebrates, being in all probability exercised through the medium of a hormone."

Mention must be made in this connection of the occurrence of gynandromorphs in which by direct cellular determination each part of the body develops independently of other parts (mosaics). Gynandromorphs should not be considered, as some authors have considered them, as contradictory to the assumption of a hormonal control of secondary sex characters, since different tissues may respond differently to the presence and concentration of hormones. The data available at present about "intercastes," i.e., intermediate forms between the normally well differentiated castes of ants caused by parasitic nematodes (190, 180), do not furnish conclusive evidence of possible participation of hormones in the development of insect castes.

Finally, the mutual relationship between the corpora allata (p. 395)

[2] I. W. Pfeiffer again found that, in grasshoppers, castration and grafting of gonads do not influence the development of secondary sex characters. (Personal communication.)

and the ovaries in adult insects (175, 204; p. 400) seems to speak in favor of a humoral rôle of insect gonads.

It thus appears that the assumption of an active rôle of sex hormones in insects is still rather inadequately supported, although it cannot be completely refuted. From the data available at present several possibilities are open for discussion. 1. Sex hormones may be present in the insect body, as was shown to be actually the case by Loewe and his co-workers (116, 117; p. 403), but they may not influence the secondary sex characters (104). Observations in parasitized insects tend to refute, however, this possibility, unless one prefers to accept Goldschmidt's (68) interpretation of parasitic castration as a type of intersexuality. 2. The negative results in most of the experimental work dealing with this problem might be explained by the fact that the operations were not complete or that, at the moment of castration or transplantation, the secondary sex characters had already been determined. This assumption is ruled out at least in the experiments of Geigy (62) who obtained complete castration by elective ultraviolet irradiation of the primordial germinal cells in the "egg" of Musca. There was no indication of an endocrine activity of the gonads, at least regarding the differentiation of secondary sex characters. 3. Sex hormones may be produced within the insect body in an organ other than the gonad itself. This is denied, for instance, by Rempel (151) but is suggested by the work of Iwanoff and Mestscherskaja (87) who, on the basis of transplantation experiments in Blatta, attribute to the fat body the formation of a sex hormone responsible for the maturation of the gonads as well as for the determination of the secondary sex characters. A second hormone, originating in the degenerating egg follicles, was claimed by the authors to infantilize the ovaries temporarily, i.e., for the period in which the cocoon is carried by the female. As similar effects are obtained with unspecific substances, the experiments as well as their interesting interpretation need further confirmation.

Thus in insects the state of affairs is still not clear and at present the question of a hormonal control of sexual functions cannot be answered satisfactorily, whereas in crustaceans, worms and molluscs the evidence available points towards an active rôle of hormones in the determination of secondary sex characters.

Gene hormones. It is known that in certain cases, mainly insects, hormonelike substances play a rôle in the development of hereditary characters. Some tissues of animals containing certain genes were found to produce and give off specific diffusible substances under the influence

of which the respective characters develop. These gene-controlled substances ("gene hormones," 109), when released from cells of the genotypes, may induce in animals which lack these particular genes a non-autonomous development characteristic for the donor cells. For reviews of these problems, so far mainly worked out by Kühn, Ephrussi, Beadle and their co-workers, the reader is referred to the papers of Ephrussi (58) and Plagge (147).

The action of gene hormones can be ascertained in various ways. One of these is by an analysis of insect mosaics. This was first done by Sturtevant (172) who in gynandromorphs of Drosophila melanogaster found, for example, the female organs to influence the color of the genetically male eyes. A second is by endocrinological methods, i.e., grafting of tissues between donors containing and hosts lacking a certain gene and vice versa, or by application of the gene hormones themselves through feeding, injection or the addition to organ anlage in vitro.

Characters developing by means of gene hormones are, for example, certain eye colors. Thus in the meal moth Ephestia there exists, except in the black-eyed wild race ($a^+a^+$), a mutant with red eyes (a a). If into a-larvae the testes of wild-type larvae are grafted, the resulting adults exhibit normally pigmented, that is, dark eyes instead of the expected red ones. One must conclude, therefore, that the implanted testis furnishes a substance which in the host modifies the development of the genetically determined eye pigment. In the red-eyed mutant the agent responsible for the formation of normal, i.e., $a^+$ or wild-type eye pigment apparently is lacking as a consequence of a genetic constitution altered with respect to one gene (a). This deficiency can be overcome, however, as the mutant retains its ability to respond to the diffusible agent. In Ephestia this substance, called "$a^+$ hormone," is produced in addition to the gonads also in the brain and ventral cord (150), in the fat body and in the hypodermis (124). Other non-autonomous characters undergoing similar somatic changes under the influence of $a^+$ hormone are the color of the skin, of the testis and of the brain. Thus an a-testis implanted into an $a^+$ host assumes the phenotype of the wild race under the influence of the $a^+$ substance circulating in the body of the host. Similarly certain eye-color characters in Habrobracon, a wasp parasitizing on Ephestia, were found to be non-autonomous (191).

In Drosophila, in view of the experimental results obtained, two eye-color hormones have been postulated, one for the character "vermilion" ($v^+$ hormone) and one for "cinnabar" ($cn^+$ hormone). Both substances

are produced in the eyes and in the Malpighian tubes, while the fat body only furnishes $v^+$. The $v^+$ substance must be considered as an active precursor in the formation of the closely related $cn^+$ substance. Both substances are contained in the $a^+$ substance of Ephestia and they were also found in corresponding substances obtained from a number of insects (112) as, for example, the fly Calliphora, the moth Galleria, and even from the crab Uca.

Gene hormones are thus neither species- nor genus-specific. It is of interest that in Drosophila pigment formation in eye anlagen could also be observed in vitro. If $v^+$ hormone was added to the culture medium, anlagen of organs lacking $v^+$ gave a similar response as in vivo (69). The gene hormones, found to be present in the body from early stages, seem to be furnished by the different organs in various amounts and at different times. The formation was found to begin in Drosophila eyes, for instance, 40 hours after puparium formation (47). The concentration in the lymph of the wild type is higher in pupae than just before puparium formation. The influence of the gene hormones is exerted at definite times during development ("sensitive" or "effective" periods). Gene hormones are maternally transmitted to the $F_1$-generation. They are stored in the organs of formation; their production is greatly influenced by nutrition (174). Quantitative studies indicate that $a^+a^+$ cells produce the same amount of hormone as $a^+$ a cells. Within certain limits the reaction obtained after injection of gene hormones is proportional to the concentration. The substance is not released from an organ, for instance a wild-type eye, before the fulfilment of the organ's own requirements ("priority effect").

Gene hormones can be extracted from the organs and, in Drosophila, they can be separated from the pupation hormone (p. 398). As they have been purified to a certain extent, some of the physico-chemical properties are known. The gene hormones are heat resistant, soluble in water, less soluble in alcohol, acetone, etc.; they are dialyzable. Toward acids and alkali they react similarly as the pupation hormone (p. 398). A substance having the chemical and physiological properties of the $v^+(a^+)$ hormone was found to be synthesized by certain bacilli in the presence of tryptophane (173, 42). This lead Butenandt, Weidel and Becker (42) to the conclusion that the $v^+(a^+)$ substance is probably identical with kynurenine, a derivative of tryptophane, which has a rather specific effect on the eye coloration of Drosophila and Ephestia. Whether this substance will prove, on further study, to be a "gene hormone" or rather a chemical precursor of the eye pigment cannot be

decided at present. For other interactions between hormones and genetical constitution see (72) and (141).

COLOR CHANGE. As in vertebrates, there occur in invertebrates two types of pigmentary reactions: 1, a morphological color change consisting in the formation and destruction of pigments, and 2, a physiological color change caused by the more or less rapid expansion or contraction of the chromatophores, which in most cases is brought about by the migration of pigment within the cell. The physiological color change is of most interest here, since it is controlled to a large extent by hormonal (neurohumoral, 132) factors.

*1. Crustaceans.* The group of invertebrates best known with respect to an endocrine control of pigment migration are the crustaceans and among them particularly the decapods. Koller (98, 99) was the first to find in Crago (Crangon) vulgaris that under certain conditions blood transfusions result in distinct reactions of the pigmentary system. He therefore assumed that a humoral agent circulating in the blood plays an important rôle in color adaptation. Later Perkins (133) in Palaemonetes and Koller (100) in Crago simultaneously described the eyestalk as the place of formation of this substance or substances (p. 392) which now are mostly referred to as "crustacean chromatophore activators" or "crustacean chromatophorotropic hormones or principles." The chromatophore activating material is produced within the eyestalk in the sinus gland, formerly called blood gland (78), as can be shown by injection of extracts or by implantation of the sinus gland into the abdomen of eyestalk-amputated crustaceans (33). The glandular structure of the sinus gland and its intimate relation to the blood system characterize it as an endocrine organ. The rôle of a second endocrine gland (X-organ) in the eyestalk is still unknown (76).

Generally humoral agents, if present in sufficient concentration, bring about color change either by preventing diffusion or by causing contraction of pigment on the one hand, or on the other hand by permitting, if absent, expansion of pigment. The same agent may, however, have the opposite effect, depending upon the species and its pigmentary apparatus. In other words, expansion of the pigment may indicate the absence of chromatophorotropic hormone in the blood (Palaemonetes, 133), or it may be a sign of its presence (Uca, 44). In most of the cases studied (for exceptions see 100, 167, 35, 198, 36, 199) there seems to exist no humoral agent acting antagonistically to the eyestalk substance (32). Consequently one phase of pigmentary activity (expansion or contraction) is hormonally induced, and the reverse phase results

passively on the basis of a property inherent in the pigment cell itself. Different chromatophores may differ in their responses to the chromatophorotropic hormones and show a certain independence from one another in a given animal (29, 30, 199) and they vary in different genera, although one type of chromatophore may be chiefly responsible for the outcome, as for instance in Uca. Pigment expansion may also be only partial or there may be in the same animal locally different responses (101). The combined reactions of the various kinds of chromatophores in response to the release of the chromatophorotropic hormones into or its disappearance from the circulation result in a state of distribution of pigment characteristic for the different species. Finally a certain degree of inconsistency of pigmentary reaction (1) and possible responses to stimuli other than the specific hormones (pp. 391–392) represent certain difficulties in the evaluation of experimental results.

In the study of crustacean color change several ways of experimental approach may be used. 1. Under different conditions of light, background, time of day, and so forth, the chromatophores show expansion or contraction. On a white background, for example, the chromatophores in Palaemonetes exhibit contraction due to the presence of eyestalk hormone (28). Certain species, for instance Uca, show a diurnal rhythm of pigment migration, considerably uninfluenced by environmental conditions, particularly by constant darkness (184). This rhythm, the mechanism of which is still unexplained, is attributed to a periodic release of hormone and not to its cyclic exhaustion and elaboration. 2. In the great majority of cases the color change in crustaceans is disturbed by the operative removal of both retinae or the coating of the eyes, as pigment migration takes place in accordance with stimuli received through the eyes (30). In a brachyuran such an operation causes expansion of melanophores as in lower vertebrates. This indicates that after eye excision, chromatophorotropic hormones are continuously released, thus also disturbing, at least temporarily, the diurnal rhythm (171, 6). The ventral half of the eye, as in certain fishes, is the decisive factor (79). Heterologous stimuli of the optic nerve, i.e., electrical stimulation or slight cauterization in eyestalk-amputated animals, or cutting of the optic nerves, have the same effect as has light on normal eyes, in a similar way as in the frog. 3. Results completely different from those just outlined are obtained after amputation of the entire eyestalks, sometimes still, but incorrectly, referred to as "blinding." Here the source of the chromatophorotropic material

is removed with the effect that, independently of the color of the background or of light conditions, for instance, the red pigment in Palaemonetes is permanently expanded. 4. Chromatophorotropic hormone can be added to the circulation by injection of organ extracts into either normal or eyestalk-extirpated crustaceans or by feeding hormone containing tissue (100). If the source of eyestalk extract is the same species or even the test animal itself, the coloration obtained is that characteristic for the presence of hormone in the blood of an intact animal (133). Therefore, the result of such an experiment as this is predictable, provided that the normal behavior of a given species is known. Qualitatively the same results are obtained, as a rule, after reciprocal injections, as for instance, of eyestalk extract from Palaemonetes injected into Uca. Tests among at least 30 different crustaceans showed that frequently eyestalks exhibit more potencies than the donor's pigmentary activity would require. Similarly successful were tests with organ extracts from sources other than crustaceans, such as corpora cardiaca, cerebral and frontal ganglia of insects (36; see also 77, 78, 90), central nervous system of Limulus (197; see also 205), neural gland of ascidians (5; pp. 401–402), sheep pituitary (p. 402).

Thus there exists no specificity regarding the efficacy of the active principles in crustacean color change. This interspecificity is also shown by the fact that crustacean eyestalk hormones act upon vertebrate chromatophores (p. 403).

As to the specificity of the hormone producing organ in crustaceans, there are, in addition to the sinus gland, few tissues to which similar effects on pigmentary distribution have been ascribed (8, 97). The "rostral organ" of Koller (100; see also 15, 32) acting antagonistically to the active material of the eyestalk seems to be confined to very few species (135, 106). Brown and co-workers (35, 198, 199) found part of the circumesophageal connectives (commissural gland) of Crago and Palaemonetes yielding a substance dispersing the dark pigment of the telson and uropods of Crago, and while white pigment is dispersed by sinus gland extracts it is contracted by an agent present in the central nervous system (36). There is indication for the presence of two different hormones in the commissural ganglia (199).

Few substances from other sources than those just mentioned are known to act upon crustacean chromatophores. In some species expansion of pigment is caused by adrenaline (15). The interesting pigmentary reactions observed after injection of distilled or tap water (8) are probably due to osmotic changes in the animal. This suggests the

possibility that the chromatophorotropic hormones affect the chromatophores by regulating primarily the salt or water balance of the animal. The occurrence of a chromatophore activator in species lacking chromatophores would thus be explained (78, 169). Sea water injections may —possibly by a dilution of hormone present in the blood—or may not affect the state of pigment distribution. Further influences affecting crustacean color change are temperature and such ions as Li, Na, K, Ca, which cause slow and irregular responses (15). A number of other agents, as, for example, different drugs, electrical current, growth substance of plants (133, 15, 129), have been found negative.

In crustaceans, eyestalk extracts show some effects other than those produced upon chromatophores, but the exact origin of the responsible substances within the eyestalk or their relation to the chromatophore activators is not known in all these cases. One of the effects concerns the pigmentary adaptation of the compound eye. In animals kept in the dark, after injection of eyestalk extracts from light-adapted specimens, a migration of the distal and the reflecting retinal pigments takes place. The resulting position is such as that typical for a crustacean kept in the light (95, 185, 49). In the dark there occurs not only diminished release or none, but apparently either less or no synthesis of the substance influencing the retinal pigments. This hormone, which is believed to be different from the chromatophore activators (78), is not genus-specific. Furthermore it was found that there exists, for instance, in Cambarus a diurnal rhythm of retinal pigment migration which continues for some time in specimens kept in the dark. This has been explained (208) by a regularly changing activity of nervous centers inhibiting the hormonc release.

Other influences attributed to the eyestalks concern the calcium metabolism (101; but see also 203), and the rhythm of the heart (183) and the musculature (91) of crustaceans. Eyestalk removal was found to cause acceleration of molting and increase in body size (34, 9, 203, 206). A favorable effect of the presence of the eyestalks or their extracts on the viability of crustaceans was claimed by Brown (31, 34), but this was not confirmed by Kleinholz (203).

When summarizing the data on the activity of the chromatophorotropic principles in the crustaceans two explanations offer themselves (5). 1. According to the unitary theory the organization of the various pigmentary effectors is responsible for the different reactions caused by one common eyestalk hormone. 2. The multiple theory claims the presence of more than one chromatophorotropic hormone in crustaceans;

at least two active substances are postulated by Kleinholz (96) to explain the known facts. A first attempt toward a physiological and chemical differentiation of two such principles within the sinus glands of seven different species was made by Brown and Scudamore (37; see also 198, 199). However, no final answer as to the number of active principles can be given so long as their chemical constitution remains unknown.

Some quantitative estimations regarding a chromatophore activator of Palaemonetes (4) suggest the following conclusions: *a*. In black-adapted animals a small quantity of hormone is present in the eyestalk. Incident light, regardless of the background, steps up the formation of hormone as compared with darkness. *b*. If, in addition, the light is reflected from a white background, a maximal hormonal release into the blood results and an increased rate of hormone synthesis takes place. The rate of color change was found approximately halved after amputation of one eyestalk (30, 45).

A method for the standardization of a crustacean chromatophore activator was devised by Abramowitz and Abramowitz (8) who used as criterion the reaction of the black chromatophores of eyestalk-extirpated Uca pugilator. The minimal amount of hormone detectable in such assays is 0.000016 $\gamma$. The thresholds of melanophore responses vary from animal to animal and "the degree and the duration of melanophore expansion are exponentially proportional to the dosage injected."

The relative effectiveness of extracts from the sinus gland and from the commissural gland (p. 391) differs with respect to different pigments (199).

The properties of the chromatophorotropic hormones may be summarized as follows. 1. Originating in distinct regions of the body the hormones, by way of the body fluid, reach the effector organs upon which they exert specific effects. 2. The action of the chromatophorotropic hormones is inter-specific (p. 391). 3. Solvents for the hormones are water (133), absolute ethanol, and methanol; they have been found insoluble in ethyl ether, chloroform, acetone, and benzine. 4. They are resistant to digestive enzymes (101), to boiling (133, 36) and to short boiling with alkali or acid, to cold temperatures (129), and to desiccation (135, 36), but they are destroyed by oxidation. 5. The hormones are readily adsorbed (3). 6. Although purification of eyestalk extracts has been performed by Abramowitz (7), the chemical constitution of the chromatophore activators, probably nitrogenous bases, is not yet known and the question of their identity with the intermedin of the vertebrate

pituitary must therefore be left open. Both materials show such close similarity in their physical and chemical properties (solubility, etc.) as well as in their physiological behavior, that they yield reciprocal results, provided they are administered in proper doses. Crustacean eyestalk extracts act upon vertebrate chromatophores (103, 125, 134, 107), and sheep pituitary and neural gland of ascidians influence crustacean color change (2, 5).

*2. Insects.* In a few groups of insects a morphological as well as a physiological color change has been observed. The best known example is the phasmid Dixippus morosus which, like some crustaceans (p. 390), shows a diurnal rhythm of coloration (184). Colored illuminated backgrounds influence the coloration of Dixippus (65, 11, 149, 88), but the effect disappears when the lower half of each eye is coated. Such animals soon become dark because of expansion of the pigment (physiological color change). Later, in addition, a formation of pigment takes place (morphological color change). Similar behavior is characteristic of an animal kept constantly on a dark background. Body regions from which the blood supply is temporarily cut off by ligatures become pale and remain so until circulation is restored. Decapitation results in paling of the whole animal (88). Transplanted pieces change their coloration simultaneously with the host (89).

From such observations and from the lack of an innervation of the cells responsible for the color change hormonal control of the physiological as well as the morphological color adaptation was concluded. Absence of the hormone in the body fluid results in concentration of pigment and in cessation of pigment formation. The source of this endocrine substance is in the head region of the animal. It is also known that the corpora allata (p. 400) play a rôle in the color responses of Dixippus (79, 139, 141), but this rôle is not yet fully understood. Denervation of the corpora allata resulted in unusual color patterns (morphological color change); reimplantation of corpora allata into allatectomized animals caused blackening of the hypodermis in the neighborhood of the implant. On the other hand, extirpation of these organs as well as of the corpora cardiaca (p. 397) had no influence on the color change in Dixippus (11, 139).

In addition to light, humidity also was found to cause pigmentary changes through hormonal action in Dixippus. On the other hand, local color reactions, brought about by temperature changes, distilled water, ether, etc., are not under hormonal control.

The effect of extracts from insects, such as Dixippus, on the chromatophores of crustaceans has already been mentioned (p. 391).

The possibility of hormonal control of the morphological pigment adaptation in certain pupae of Lepidoptera (25, 26) and of the physiological color change in the air-sacs of Corethra larvae (118) has been discussed by Giersberg (66) and others.

Concerning possible hormonal factors in the color adaptation of cephalopods see Sereni (164) and p. 401.

Hormonal control of insect development. Since Kopeć (105) showed for the first time that pupation in an insect (Lymantria) is brought about by a humoral agent, the existence of hormones involved in molting, pupation and metamorphosis has become a well established fact. The presence or absence of such hormones was demonstrated in a comparatively simple way by *a*, blood transfusions; *b*, by interruption of the circulation by means of ligatures; *c*, by injection of various extracts, and *d*, by transplantation of certain organs. In experiments of this kind the "critical period" was found to be of importance, i.e., a distinct phase preceding ecdysis, pupation, or emergence of the adult, which represents the time of hormone release (p. 398). With such methods an endocrine control was ascertained with respect to molting in Lepidoptera (18, 146) and Hemiptera (192), and to pupation in Lepidoptera (105, 110, 146) and Diptera (60, 18, 19, 123, 16, 52); see also (63). Similarly a factor governing the differentiation of the imago was found to originate in the anterior body region of Lepidoptera (71, 18, 142, 143, 209), of Diptera (19, 20; see also 92) and of Hemiptera (192). The hormonal nature of this factor for which good evidence has been furnished by the work of Piepho on Lepidoptera (142, 143), and others, has been questioned, although not disproved for Drosophila (21).

In recent years, with the development of a more delicate technique, viz., extirpation and implantation of glands in various developmental stages, more detailed results were obtained. At present the interest centers around such questions as the analysis of different developmental steps, the origin of the hormones involved in these partial reactions, the chemical nature of the hormones and their mode of action in correlation with the effector organs. In an evaluation of the data now at hand it should be borne in mind that they refer to representatives of different types of insect development, i.e., Holometabola and Hemimetabola.

So far mainly two organs of internal secretion, the corpora allata and the cerebral ganglion, are known to play an important rôle in the molting and metamorphosis of insects. Kopeć (105), and later Kühn and his co-workers (110, 142, 146), demonstrated by two procedures that

in Lepidoptera the supraesophageal ganglion (brain) furnishes a substance causing pupation. 1. Extirpation of the brain after the last larval molt prevents pupation, provided that the operation is performed before the critical period. 2. Reimplantation of a brain into such an animal restores the capacity for pupation. A possible rôle of the frontal ganglion in pupation has been discussed also (23).

In the hemimetabolous Hemiptera the development is characterized by the occurrence of a number of molts, the last of which is coupled with metamorphosis and leads to the adult stage. Here a part of the nervous ganglia produces a "molting hormone," as was conclusively demonstrated by Wigglesworth (195). If, in the bug Rhodnius, the central mass of the protocerebrum from a donor in the critical period is implanted into decapitated nymphs, molting occurs. Other parts of the central nervous system, the corpora allata, the fat body, etc., give negative results.

Wigglesworth showed in Rhodnius, in addition to the molting hormone, the action of an antagonistic principle, the "inhibitory hormone" which originates in the corpus allatum (192, 195). There is good evidence that this factor, in all but the last larval instars, prevents metamorphosis and favors the occurrence of further nymphal molts. Thus in collaboration with the molting hormone the inhibitory hormone controls the rate of development. The experimental data obtained not only in Hemiptera and Orthoptera, but also in Lepidoptera, are in agreement with this assumption.

1. In Rhodnius (Hemiptera) removal of the head during the critical period, i.e., excluding the inhibitory factor just before its release into the circulation (p. 399), causes metamorphosis to occur earlier than normally. Extirpation of the corpora allata in the third or fourth nymphal instars of Dixippus (Orthoptera) decreases the number of subsequent molts and thus causes accelerated maturation (137). Similarly in early larval instars of Bombyx (Lepidoptera) allatectomy leads to suppression of molts and to precocious pupation and results in normal adults (24).

2. Removal of the corpora allata in the last nymphal instar of the Orthopteran Melanoplus (136) or in the last larval instar of the Lepidoptera Bombyx (24) and Ephestia (110) has no influence on the final development. In these cases no inhibitory factor is active and the removal of its source, therefore, does not show an effect.

3. Implantation of corpora allata from a young caterpillar into one which is ready to pupate, i.e., adding the inhibitory factor where it is

normally absent, prevents the onset of metamorphosis in certain moths (110, 146, 144, 24). In grasshoppers (140) or bugs (194) the implantation of corpora allata may thus also cause several supernumerary molts.

In this way the function of the corpora allata appears to be "inhibitory" with respect to the development of adult characters in representatives of three orders of insects, viz., Hemiptera, Orthoptera, Lepidoptera.

In a representative of the Diptera, Drosophila melanogaster, Hadorn (72) proved that puparium formation is brought about by an agent originating in the ring-gland (Weismann's ring). Puparium formation, which is greatly retarded or fails to occur in lethal larvae of the mutant lgl and in certain hybrids, can be accelerated by implantation of genetically normal ring-glands. This effect cannot be brought about by brain implants. Furthermore, within certain limits puparium formation in normal larvae takes place the sooner the more mature ring-glands (from one to three) are implanted (73). This rôle of the ring-gland was confirmed for Calliphora by transplanting the organ into posterior larval fragments (16). Correspondingly, extirpation of the gland or section of its nervous connections in Calliphora prevents puparium formation and arrests the growth of the imaginal buds (40). In Drosophila, however, the ring-gland does not influence imaginal development (73). Morphologically the ring-gland is the homologue of both corpus allatum and corpus cardiacum. The histological appearance of the two types of glandular elements in normal as compared with lethal Drosophila larvae (154; p. 398) suggests that the larger gland cells which now are believed to represent the corpus cardiacum (201; cf. 39) are the possible source of the "pupation hormone."

To summarize: In three (Hemiptera, Orthoptera, Lepidoptera) of the four orders of insects studied the corpora allata are known as the source of a hormone inhibiting imaginal differentiation. In the Diptera a principle causing puparium formation is assumed to be furnished by a glandular tissue which represents the corpus cardiacum. The endocrine rôle of the supra-esophageal ganglion has been demonstrated in two orders, i.e., in Lepidoptera where it controls pupation, and in Hemiptera where it furnishes a hormone, that, in coöperation with the inhibitory factor (p. 396), controls molting.

Whereas the glandular character not only of the corpora allata, but also of the corpora cardiaca has been known for some time (192, 137), the concept of an endocrine rôle of the nervous system has been accepted with considerable hesitation, in spite of the fact that the occurrence of

nerve cells with an undoubtedly glandular character has been demonstrated in invertebrates as well as in vertebrates (155). That, in the insects studied, such neuroglandular cells are actually the source of the hormones found in the physiological experiment is strongly suggested by the following histological findings: neurosecretory cells occur not only in the larval brain of Lepidoptera (51), such as Ephestia, but also in Rhodnius where they are located in the pars intercerebralis (79), i.e., the very region of the nymphal brain yielding the molting hormone in the experiments of Wigglesworth (195; for further literature on neurosecretory cells in insects see 153, 200; concerning the endocrine rôle of the central nervous system in color change, see p. 391).

The data just reviewed may seem to be discordant. It should be kept in mind, however, that insect development is a complex mechanism with more factors involved than those here outlined, and that the various orders differ from one another to a considerable extent. Furthermore, it is probable that additional endocrine factors will be discovered which will lead to a better understanding of a field that has, as yet, only been touched upon. Nevertheless, some generalizations can be made now with regard to the properties and the mode of action of the hormones controlling insect development.

1. It seems rather certain that more than one principle is involved in the different steps of insect development. To what extent these substances differ from one another can hardly be decided so long as their chemical constitution is still unknown.

2. Extracts made from and tested in Calliphora led to a certain purification of the pupation hormone and to its separation from the $a^+$ substance (p. 387), with the result that some of the properties of the pupation hormone are known (16). It is soluble in water, alcohol, butanol, acetone, etc.; insoluble in chloroform, petroleum ether, etc.; resistant to heat and acids but not to alkali. It is readily dialyzable.

3. The hormones regulating insect development are not specific with respect to the genus (193, 73, 143), or even to the order (16).

4. The proper action of the hormones depends on the state of the glands which furnish them, as well as on the state of the reacting organs and tissues. Hormones are produced and released only by mature glands. Thus in the mutant lgl of Drosophila, for instance, the inability to pupate (p. 397) was found to be due to a genetical deficiency to develop fully grown ring-glands which lack the power to produce a sufficient amount of pupation hormone (154). The release of hormone takes place gradually during the "critical period" (p. 395) which, in

Calliphora, for example, is 8 to 12 hours before puparium formation (16). In a case where, as in Rhodnius, two hormones interact, the factor inhibiting metamorphosis was found to be given off later than the molting hormone (192).

5. Regarding the reaction of the effector organs to the hormonal stimulus, two points are of importance. *a.* The reacting organs (hypodermis, Verson glands, etc.) must be in a physiological state of preparedness in order to guarantee proper responses (19, 146, 73, 145). *b.* Within this limit the reacting material exhibits a considerable independence. Grafted organs are capable of undergoing developmental changes simultaneously with their host and to a certain extent irrespective of their own ages. This may lead to supernumerary molts or to accelerated development of the implants (119, 20, 111, 195). The longer before the critical period the stimulus occurs, the less sensitive is the reacting material to a given hormone concentration (16). The response to the pupation hormone is characterized by well defined steps, viz., onset of mitoses, separation of larval cuticle from hypodermis, secretion of exuvial fluid. The latter two phases may be suppressed if the hormone supply is insufficient (111). But once a sufficient stimulus for pupation or for imaginal differentiation has been received, completion of the process may take place independently of the subsequent presence or absence of the stimulating hormone (145, 20).

6. The action of the hormonal factors governing insect development is to a certain extent quantitative. Small amounts of hormone may yield incomplete metamorphosis (193) or partial pupation (110, 111, 142, 16; see also p. 393). In such cases certain regions of the hypodermis prove to be more sensitive to low hormone concentrations than others, an observation which has been connected with the important rôle oxygen plays in the differentiation of insect tissues (21, 16).

7. Various environmental factors interact with those of hormonal nature in the regulation of insect development. In the mealmoth, Ephestia, the release of the pupation hormone can be inhibited by low temperatures. The resulting "permanent larvae" pupate when brought back to room temperature (E. Caspari, personal communication). Combinations of pupal and adult characters may be obtained in individual silkworm pupae by the application of temperature gradients (209). For other examples see, for instance, the papers of Mellanby (123) or Bodenstein (196).[3]

[3] Regarding hormonal interactions between ovary and molting rhythm in isopods (crustaceans) see Haemmerli-Boveri (74).

Aside from their rôle in insect development *additional endocrine functions of the corpora allata* may be briefly discussed. Their presence is necessary for the attainment of sexual maturity in some species (193, 175), but not in others (137).[4] In the grasshopper, Melanoplus differentialis, the corpora allata are necessary for the deposition of yolk and for the production of secretion by the oviducts. After reaching the proper stage of maturation the oviducts respond with secretion to the presence of corpora allata, irrespective of whether these glands are grafted or in situ, from males or females, from nymphs or adults (136, 204). Larval ovaries transplanted from Drosophila melanogaster into Drosophila funebris fail to develop yolk unless transplanted together with melanogaster-ring-gland (207; p. 397). Considering the results of Pfeiffer (136) it is possibly the allatum-component of the ring-gland which furnishes a substance necessary for the egg development of Drosophila melanogaster. Whether a species-specificity of a hormonal action really exists to such a degree needs further investigation. The corpora allata influence color change (p. 394), growth regulation (119, 141, 22) and regeneration (140). Removal of these glands in an early developmental stage causes degenerative processes in certain tissues such as the fat body, the musculature and the nervous system, as well as abnormal growths interesting for their similarity to certain tumors in vertebrate pathology. Reimplantation of the glands restores normal tissue growth (139). Actions of the kind described suggest that the rôle of the corpora allata consists in a general influence on metabolism (140), but the question is how far this concept may explain all of the special effects attributable to the glands.

The versatile character of the functions of the corpora allata in influencing color change, sexual maturation, regulation of growth, etc., might suggest a comparison of this gland with the hypophysis of the vertebrates. Although the concept is highly interesting, it should be kept in mind that a homology between the two organs should, by no means, be constructed from a comparison of this kind.

Except for the corpora allata, the corpora cardiaca (pp. 395, 397; see also 136), and the cerebral ganglia there occur in insects a few organs of

[4] The honey bee represents a special, although still somewhat controversial, case. Sexual maturation of the queen as contrasted with the worker bee is known to depend on the special food (royal jelly) provided during larval life. In this royal jelly an active material has been found which has a certain gonadotropic activity as demonstrated by its effect on the reproductive system of rats (85) and of Drosophila (176).

possible endocrine nature: the "pericardial glands" and the "ventral glands" (139, 141), the fat body (p. 386) and the silk glands (Umeya, see 80). The Verson glands, oenocytes, and synoenocytes are now believed not to belong in this category of organs (111, 80).

In addition to the reported data there are a number of more or less well established cases of hormonal actions in various invertebrates. Thus, for instance, tho *internephridial organs* which form a part of the nephridia of the marine worm Physcosoma lanzarotae, show a striking similarity to the interrenal body of the vertebrates (81). Total extirpation of these glands leads to disturbances which resemble those of adrenocortical insufficiency in man (Addison's disease) and, within a few days, to death of the operated worms. Reimplantation of internephridial tissue or parabiosis with a normal animal brings about recovery. The internephridial cells exhibit various stages of a secretory cycle and their granular inclusions are similar to those of the adrenal cortex. These data do not hold true for Physcosoma japonicum, a species which survives total extirpation of the nepbridia for more than two weeks (102).

Regarding hormonal activities in molluscs mention has already been made of the possible endocrine significance of the gonads (p. 384). Further organs of internal secretion are in all probability the *posterior salivary glands* and the branchial glands of the cephalopods (164, 126; see also 94). Similarly "purpurin" from the hypobranchial glands of the snail Murex (55) and a pressor substance related to "adrenin" in the mantle of Purpura (152) have been described as substances of hormonal character.

Further examples of supposedly endocrine functions which, however, before they can be included as true hormonal activities need further investigation, are found in the works of Hanström (80) and Koller (102).

In *tunicates*, the forerunners of the vertebrates, two organs show homologies with endocrine glands of the vertebrates. 1. The *endostyle* is commonly considered as a primitive thyroid. This gland, however, has no effect on amphibian development (168), and the acceleration of ascidian metamorphosis by vertebrate thyroid (181) is probably not due to thyroxine (70). 2. The *neural gland* (subneural gland) of the ascidians corresponds to the posterior lobe of the vertebrate pituitary. This is demonstrated not only by anatomical considerations but by pharmacological assays. The neural gland contains the oxytocic principle of the pituitary (tests with uterus of the rat and the guinea pig; 41, 12). The pressor effect is obtained upon injecting neural gland ex-

tracts intravenously into cats (12). A chromatophore-expanding action may be observed, for instance, in the frog (12, 57). There is no gonadotropic principle in the ascidian neural gland (17). The functions of both these glands in the tunicate organism are still unknown; the neural gland possibly regulates, through the oxytocic principle, the discharge of the sexual products (86).

An extensive literature (187, 102, 80) deals with the mutual *relationships between invertebrate and vertebrate endocrines.* Whether some of the invertebrate hormones are identical with known vertebrate hormones, cannot be decided so long as the chemical constitution of invertebrate hormones is so imperfectly known. However, irrespective of their chemical relations, vertebrate hormones are known to exert an influence on invertebrates and vice versa.

The response of invertebrates to *thyroid,* as tested by a number of investigators, is discussed by Schneider (156), with the conclusion that in protozoans and in fertilized eggs of several invertebrates "thyroid apparently accelerates both anabolism and katabolism resulting in an increased rate of cell division and excretion. Thyroxin, on the other hand, seems to affect only the katabolic processes in the cell, hence the accelerated rate of excretion and the slightly depressed rate of cell division." Regarding an influence on the development of arthropods the data are ambiguous, but at least in one carefully studied case a clear effect was observed. Thyroid-fed flour beetles (Tribolium) show acceleration of larval development and increase in growth. In a diminishing degree this effect is transmitted by the female to several generations fed on thyroid-free diet (157–161).

*Parathyroid* (Parathormone Collip-Lilly) is said to have a certain influence on the calcium metabolism of crustaceans (131; see also p. 392), but there is as yet no definite proof for a specific action of *thymus* on an invertebrate organism.

Growth, molting, and regeneration in isopods (Asellus) are reported to be considerably accelerated by *pituitary* extracts (75). Anterior lobe shows a gonadotropic influence, viz. accelerated maturation of eggs, in the polychete worm Lycastis (Feuerborn, see 80). Posterior pituitary is said to cause maturation in female insects (87). Color change in crustaceans is brought about by intermedin from vertebrate hypophysis (p. 391).

The *adrenal cortex* was found to exert a favorable influence on growth rate, maturation, and fertility of crustaceans (82, 46) and on the growth of snails (83). The effect of *adrenaline* on protozoans (186), i.e., an increase in the viscosity of the cytoplasm which is correlated with the calcium content of the medium, is of interest in view of general cellular physiology. Furthermore, adrenaline influences the vascular system of annelids (61) and it produces hyperglycemia in annelids, molluscs, and crustaceans (120), an effect which is considered by some authors as unspecific (93). Regarding the effect of adrenaline on color change see p. 391. As in vertebrates, adrenaline increases the tone and contractility in the musculature of worms, arthropods, and molluscs, causing such reactions as acceleration of the heart rhythm or, in cephalopods, pigment expansion (164, 13, 130, 108, 182).

There appears to be no known case of a specific action of vertebrate *gonadal extracts* on an invertebrate.

*Insulin* is said to have an influence upon the metabolism of protozoans, comparable to that in the mammalian organism (38). The chromaffinity found in certain ganglion cells of the leech (see below) disappears under the influence of pancreatic hormone (Poll, see 187).

Mammalian *secretin* stimulates the hepatopancreas of Octopus and an analogous substance extracted from this mollusc nets on the dog (113).

Otherwise comparatively few data suggest an effect of invertebrate endocrines upon vertebrates. The close similarity between the *chromatophorotropic hormones* of crustaceans and lower vertebrates has already been dealt with (p. 391). Furthermore, *estrogenic substances*, tested in castrated mammals, were found in almost all groups of invertebrates: protozoans (170, 14), coelenterates (162), worms (170), echinoderms (170, 116, 54), and molluscs (170). With few exceptions all results recorded for arthropods were positive, i.e., for several representatives of insects (170, 116, 117, 162), spiders (170, 162), scorpions (170), and crustaceans (54). See also footnote, p. 400. The seminal vesicles of castrated rats are said to become enlarged after injection of macerated seminal vesicles from earthworms (202). Finally, *adrenaline*, or at least a closely related substance, from invertebrates such as protozoans, annelids (chromaffine nerve cells), molluscs and insects (187, 188) was tested in vertebrates with positive results. Regarding tunicates see pp. 401–402.

The results of the experiments of which examples have been quoted above should be viewed critically. It must be kept in mind, for instance, that when feeding endocrine substances or adding them to culture media, the amount actually taken up by the animals is difficult to control. In some cases these substances are known to be quickly decomposed within the test animal before they can exert their influence, and the effects observed may be due to their nutritional rather than to a specific hormonal effect. A depressing action, on the other hand, may not rarely be explained by the toxicity of the material, administered in too high concentrations. Recently the importance of proper dosage was again emphasized by Schneider (156). In an evaluation of the effects of vertebrate endocrines on such functions as development and growth, fertility, metabolism, or regeneration, the normal physiological behavior of the tested invertebrates and its variability is not always sufficiently taken into consideration. Thus undoubtedly many of the reactions reported are unspecific, particularly if they are of an indefinite nature and if they are similarly observed after the administration of different endocrines. At present only those cases in which the responses are comparable with those known in the donors seem to indicate specific actions of vertebrate hormones on invertebrates and vice versa.

By way of final summary, the study of endocrine organs and their

functions is no longer restricted to vertebrates. Whereas the question of an active rôle of sex hormones in invertebrates is still controversial and the true hormonal nature of gene-controlled substances has yet to be confirmed, the rôle of endocrines in color change of arthropods and in development of insects is well established. The physico-chemical properties of invertebrate hormonal substances as well as their physiological actions are comparable with those of corresponding vertebrate materials. This is demonstrated by the fact that vertebrate and invertebrate endocrines may replace each other in certain actions. It should be kept in mind, however, that such physiological relations do not indicate anatomical homology of any one of the endocrine glands of invertebrates with those of vertebrates.

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# ORGANIC ACID-SOLUBLE PHOSPHORUS COMPOUNDS OF THE BLOOD

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This review is concerned mainly with the functional significance of the organic acid-soluble phosphorus compounds of blood. The inorganic phosphorus of the blood also is dealt with insofar as problems concerning the inorganic and organic compounds are closely interrelated. More complete discussion of the chemistry and general metabolism of these and related compounds in blood, muscle and other tissues may be found in the reviews of Kay and Byrom (74), Peters and Van Slyke (105), Kay (73a, b,) Schmidt and Greenberg (122), Robison (114, 115), Nissen (103), Lohman (88), Baumann and Stare (14). Recent investigations in which radioactive phosphorus has been used for the study of these compounds have been reviewed by Greenberg (44) and by Hevesy (60).

Those reviews cite evidence aptly described by Kay (73a) as "sometimes sound, sometimes not quite so sound," that the following acid-soluble phosphorus compounds are present or may be formed in blood: inorganic phosphates, hexose mono- and diphosphates, triosephosphates, adenylic acid, adenosinetriphosphate, phosphopyruvic acid, diphosphoglyceric acid, diphospho-$\alpha$-ketotrihydroxyadipic acid, cozymase, (diphosphopyridine nucleotide), triphosphopyridine nucleotide, and cocarboxylase. To this list was added recently phytic acid, present in avian blood corpuscles (Rapoport, 108).

Studies of the organic phosphorus compounds in blood in different physiologic and pathologic conditions have been limited necessarily to those substances for which convenient analytical procedures have been devised. Limitations of methods account for the fact that existing knowledge of variations in the distribution of acid-soluble P compounds in the blood in disease is practically limited to the inorganic and total acid-soluble P, adenosinetriphosphate, and diphosphoglycerate. Methods in current use are cited here only briefly, since they have been discussed extensively in the above mentioned reviews.

METHODS. The separation of phosphorus compounds of the blood according to their solubility in acid protein precipitants such as trichloroacetic and picric acids apparently was first made by Greenwald (45). The method which appears to be the most used in current investigations for the determination of inorganic P and total P in the deproteinized filtrates of blood is that of Fiske and Subbarow (39), but a host of other methods may be found cited in the reviews listed in the first paragraph of this paper.

Various methods have been devised for the fractionation of the organic phosphates. Kay and Robison (75) distinguished two fractions of the organic acid-soluble P, respectively hydrolyzable and non-hydrolyzable by bone phosphatase. Another method of fractionation, introduced by Lohmann (86) and first applied to blood analysis by Bomskov (15), was based on differences in the rate of hydrolysis of various phosphoric esters by mineral acids. Several fractions can be determined more or less accurately by this procedure, but knowledge of the nature of the esters thus determined is a prerequisite. The fraction most easily split is the so-called pyrophosphate, liberated by 7 minutes' boiling with N HCl or $H_2SO_4$; this represents two-thirds of the adenosinetriphosphate P. If such acid hydrolysis be continued 150 minutes, other phosphoric esters, including a large part of the adenylic acid, are decomposed. Approximately two-thirds of the organic acid-soluble P, remaining unhydrolyzed after 150 minutes, may require 48 hours for complete hydrolysis; this is usually designated the non-hydrolyzable fraction, and is composed of diphosphoglycerate and other compounds. There is of course some overlapping between these fractions.

Fractionation of the phosphoric esters by enzyme and acid hydrolysis appear to lead to fairly similar results. Kerr and Antaki (78) determined the breakdown of organic phosphates in several species of blood by bone and kidney phosphatases and by acid hydrolysis. Their results indicated that adenosinetriphosphate appeared to be the principal substrate of phosphatase, although in pig and dog bloods an unidentified compound contributed significantly to the phosphate liberated by the enzyme. Warweg and Stearns (134) determined the velocity constants for enzyme and acid hydrolysis of the organic phosphates in human blood filtrates and of pure phosphoric esters, with results indicating that the so-called non-hydrolyzable fraction of the organic acid-soluble P corresponded with pure diphosphoglycerate isolated from pig blood.

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Studies of the organic phosphorus compounds in blood in different physiologic and pathologic conditions have been limited necessarily to those substances for which convenient analytical procedures have been devised. Limitations of methods account for the fact that existing knowledge of variations in the distribution of acid-soluble P compounds in the blood in disease is practically limited to the inorganic and total acid-soluble P, adenosinetriphosphate, and diphosphoglycerate. Methods in current use are cited here only briefly, since they have been discussed extensively in the above mentioned reviews.

of the per cent cell volume determined by a suitable hematocrit method, and the organic acid-soluble P in the plasma usually is not taken into account. Constituents of the white cells are of course included in analyses of whole blood.

The amount of inorganic P normally present in blood cells has been the subject of considerable debate and conflicting opinions (Peters and Van Slyke, 105; Halpern, 57; Nissen, 103; Freudenberg, 41). Normally the concentration of inorganic P in the cells is considerably less than in the plasma, but its distribution does not correspond with the distribution of other diffusible ions such as $H^+$, $Cl^-$, and $HCO_3^-$, between the cells and serum. The concentration of inorganic P in the blood cells is probably governed mainly by factors which affect the rates of synthesis and decomposition of the phosphoric esters, rather than factors which are believed to govern the Donnan equilibrium (127). Thus the concentration of inorganic P in the cells in different conditions has been found to vary between 0 and values considerably higher than those found in the corresponding plasma.

The question of the passage of phosphorus between cells and plasma has been somewhat clarified by recent studies in which "labelled" radioactive phosphorus ($P^{32}$) has been used to trace the turnover of phosphorus in the blood. Hevesy and Aten (61) studied the rate at which labelled phosphate ions added to the plasma of rabbit's blood penetrated the corpuscles, and obtained evidence suggesting that "the penetration of the labelled inorganic phospbate into the corpuscles is a comparatively slow process while the process in which the labelled inorganic phosphate is incorporated into the easily hydrolysable organic compounds inside the corpuscles is a fast one." Eisenman and co-workers (27) also used radioactive isotopes to study the permeability of erythrocytes *in vitro* to potassium, sodium, and phosphate ions. They found evidence of active entry of the labelled $P^{32}$ into the blood cells and of its synthesis into organic phosphates, and of inhibition of this process at low temperatures. Inhibition of the transfer of inorganic P between cells and plasma by low temperatures was also discussed by others (57, 127). Eisenman and co-workers (27) concluded from their experiments that the transfer of phosphate across the cell membrane was associated with enzymatic processes rather than with diffusion. It should be noted, however, that diffusion of phosphate is not excluded by such findings, and that the diffusion gradient might be affected by rates of synthesis and decomposition of phospboric esters within the cells.

*Species.* Studies of the distribution of phosphorus compounds and of phosphatases in the bloods of different species of animals have yielded data which are of considerable interest in connection with evolutionary relationships among the species, as well as pointing to possible differences and similarities in the phosphorus metabolism of the various species.

Kay (70) reported studies of the distribution of acid-soluble P in the bloods of 11 mammalian species. His data on the concentrations of organic acid-soluble P in the blood cells correspond with data similarly determined later by other investigators, and his values for the fraction of the ester P hydrolyzed by bone enzyme correspond roughly with the concentrations of adenosinetriphosphate found by others in the bloods of those 11 species. Kerr and Daoud (80) and Kerr (77) determined the concentrations of inorganic P, adenosinetriphosphate, and organic acid-soluble P in the bloods of 28 species of vertebrates; and McCay (95), the inorganic P and organic acid-soluble P in the bloods of 3 other lower vertebrates.

Recently we reported (112) data on the distribution of inorganic P, organic acid-soluble P, adenosinetriphosphate, diphosphoglycerate, and phytic acid P in the bloods of 46 vertebrates: 22 mammalian, 12 avian, 6 reptilian, 4 amphibian, and 2 species of fish. In most of the mammalian bloods the concentration of organic acid-soluble P in the cells was between 50 and 100 mgm. per 100 cc., with a high proportion (usually one-half) of phosphoglycerate. In a small group of ungulates (beef, sheep, goats, deer) the concentration of organic acid-soluble P in the cells was much lower, 9 to 15 mgm. per 100 cc., with only traces of phosphorylated glyceric acid. (See discussion of age differences, p. 415.) In all bird bloods the concentration of organic acid-soluble P in the cells was high, 90 to 135 mgm. per 100 cc., with a large proportion of phytic acid P, 49 to 87 mgm. per 100 cc. The distribution of phosphorus in the bloods of the avian species appeared to be more uniform than that found among different species of other classes studied. The avian species also are known to show relatively few differences in anatomic structure when compared with the structural diversity found among the members of other classes of animals. The data on 6 species of reptiles indicated a great diversity in the distribution of phosphorus in the bloods of this class. In snake blood cells the concentration of organic acid-soluble P was high with a large proportion of adenosinetriphosphate. In alligator blood cells the concentration of organic acid-soluble P was low. Turtle blood alone among all species other

than avian contained phytic acid. Turtle blood also was found to have a distribution of phosphatase and phytase similar to that found in bird bloods (unpublished work). Such findings suggest a very old phylogenetic origin of phytic acid, probably among the primitive reptiles. Phosphoglycerate was not found in the bloods of any species which normally have nucleated erythrocytes. Adenosinetriphosphate was found in widely varying concentrations in the blood cells of all species.

*Sex.* There appears to be no significant difference in the distribution of acid-soluble P between the bloods of males and females of any species. Kay and Byrom (74) found practically identical concentrations of organic phosphates in the blood cells of normal men and women.

*Age.* Although the level of inorganic P in the blood is generally recognized to be higher in young animals than in older ones, only a few figures are available on changes in the organic acid-soluble P compounds of the blood at different age periods in different species of animals.

Young rats have considerably higher concentrations of inorganic P, organic acid-soluble P, adenosinetriphosphate, and diphosphoglycerate in the blood cells than older rats. These concentrations fall progressively until adult levels are reached around the sixth week of life (Kay, 72; Rapoport and Guest, 109).

Green and Macaskall (43) and Malan (91) have reported high concentrations of organic acid-soluble P and of potassium in the bloods of lambs and calves, compared with concentrations found in the bloods of grown sheep and beef. We also have found relatively high concentrations (around 60 mgm. per 100 cc.) of organic acid-soluble P in the blood cells of calves during the first week of life, with half of this accounted for in the phosphoglycerate fraction (unpublished work). This was in contrast with the cells of adult beef blood which have a low concentration of organic acid-soluble P with only a trace of phosphoglycerate. Such data might indicate that the blood cells in the young of all mammalian species have similar high concentrations of phosphorus and potassium, and that the great differences found between the blood cells of adult individuals of various mammalian species are due to secondary developments of ontogeny.

Anderson and Elvehjem (3) reported lower levels of total acid-soluble P in the whole blood of mature dogs than of young dogs, but no values for the volume of cells in these bloods were included in the report. Nissen (103) reported extensive studies of the distribution of phosphorus in the whole blood of several species, with regard to age, but gave no data on the volume of cells in the blood.

Stearns and Warweg (128, 129) found the average values for ester P in the blood cells of infants to increase somewhat from birth through the first year of life and thereafter to decrease gradually in the succeeding years of childhood.

*Types of cells.* Except for a few studies of reticulocytes, little attention appears to have been paid to the distribution of acid-soluble P in blood cells other than mature erythrocytes. Data concerning human reticulocytes have been reported by Kay (71) who studied the distribution of phosphorus in the bloods of patients with splenic anemia, acholuric jaundice, hemolytic icterus during crises, and pernicious anemia following treatment with liver extract. In such bloods the concentration of organic acid-soluble P in the cells was greatly increased, to as high as 90 mgm. per 100 cc. There was indirect evidence also that nucleoprotein, generally believed to be absent in mature erythrocytes, was present in these bloods in amounts roughly proportional to the reticulocyte count.

We have determined the distribution of acid-soluble P in rabbit blood in which practically 100 per cent of the erythrocytes showed reticulation following repeated injections of the rabbits with phenylhydrazine (unpublished studies). The concentration of organic acid-soluble P in the cells of such blood was high, and the increase was largely accounted for in the adenosinetriphosphate fraction which rose from initial levels of between 10 and 14 mgm. per 100 cc. to above 30 mgm. per 100 cc. The high concentrations of adenosinetriphosphate in the cells appeared to be correlated with high values for mean volume, mean diameter, and amount of reticulation—characteristics of immature cells. While the origin of the increased amounts of adenosinetriphosphate is unknown it appears likely that adenylic acid may be derived from nucleic acid of the disintegrating nucleus.

Henriques and Ørskov (59) found an increased concentration of magnesium and potassium in the blood cells of rabbits and dogs with high reticulocytosis following injections of phenylhydrazine. We confirmed these findings in the unpublished experiments referred to in the preceding paragraph. In the reticulocytes a high concentration of adenosinetriphosphate and of magnesium possibly may be linked with certain known characteristics of the metabolism of these cells; namely, a high glycolytic rate compared with that of mature erythrocytes and a vigorous aerobic metabolism that is absent in mature erythrocytes (131, 34). Adenosinetriphosphate participates in both aerobic and anaerobic metabolism of blood and tissue cells, and magnesium has

been shown to be essential to the functioning of adenosinetriphosphate as a coenzyme (see review by Kay, 73). Also, the porphyrin found to be present by Watson (135) may be concerned with the aerobic metabolism and thus linked indirectly with the metabolism of adenosinetriphosphate in the reticulocytes. The finding of increased concentrations of potassium, magnesium, and phosphorus in reticulocytes suggests a fundamental similarity among immature blood cells of various mammalian species, in contrast with the great differences in composition of mature blood cells of these species.

Byrom and Kay (21) reported a few observations on the distribution of acid-soluble P in the leucocytes of the bloods of 3 normal persons, and of patients suffering with leukemia. The concentration of organic acid-soluble P in the leucocytes of these bloods ranged from 59 to 84 mgm. per 100 cc., with a high proportion of enzyme hydrolyzable P. We have found no other studies of organic acid-soluble P in leucocytes reported in recent literature.

*Glycolysis.* The early literature on relationships between phosphorus compounds of the blood and glycolysis is reviewed in articles cited in the first paragraph of this paper, and by Lundsgaard (89), Roche and Roche (118) and Guest (46). The early studies yielded evidence that the maintenance of a normal level of adenosinetriphosphate and other phosphoric esters in the blood was due to their continued synthesis and decomposition during glycolysis (Barrenscheen and co-workers, 10, 11, 12; Meyerhof, 97; Engelhardt and co-workers, 29, 31, 33; Roche and Roche, 118). A new approach to the problems of blood glycolysis was afforded by the work of Embden, Meyerhof, and Parnas who formulated a theory concerning the dismutations of phosphorus compounds connected with anaerobic breakdown of carbohydrate in muscle and yeast (Meyerhof, 98; Needham, 101; Parnas, 104). Roche and Roche (119) found the Harden-Young ester (fructose diphosphate) but not glucose to be metabolized by blood hemolysates. Dische showed that glucose was phosphorylated in blood only through the agency of adenosinetriphosphate and that fructose diphosphate and triosephosphate were intermediary products formed during glycolysis. Dische was the first to suggest the essential rôle of pyruvic acid in the glycolytic process and the coupling of the oxidation-reduction reactions through which phosphoglyceric acid is formed with esterification of inorganic P and adenylic acid. He stressed the apparent self-regulatory character of the reactions comprising the glycolytic cycle in blood (24). Several investigators (Barrenscheen and Beneschovsky, 9; Schuchardt and

Vercellone, 123; Braunstein, 16) found that monophosphoglycerate was readily attacked by blood hemolysates with the liberation of pyruvic acid and formation of phosphopyruvic acid; but that diphosphoglycerate was not decomposed by blood hemolysates. Later, however, diphosphoglycerate was found to be decomposed by intact blood cells *in vitro* (110).

Elsewhere (51) we have presented a simplified diagram plausibly representing the principal steps of the glycolytic cycle in blood, as suggested by the theories of Embden, Meyerhof, Parnas, Dische and others.

In forming diphosphoglycerate as an intermediary product of glycolysis the blood cells differ from cells of other tissues where monophosphoglyceric acid is known to be formed in the anaerobic breakdown of carbohydrates. Moreover, little is known concerning the intermediate steps in the synthesis and decomposition of diphosphoglycerate in the blood cells, compared with present knowledge of the reactions involving monophosphoglyceric acid. Jost in 1927 (68) suggested that diphosphoglycerate probably played an important rôle in blood glycolysis, but more definite evidence of this than he presented was lacking until recently. Using radioactive phosphorus, Hevesy and co-workers (61, 62) found that the diphosphoglycerate normally present in blood was constantly renewed at a rapid rate *in vivo* and *in vitro*. In unpublished studies of 10 species of blood, we have found that when blood cells were incubated in the presence of NaF and pyruvate the concentration of phosphoglycerate increased in all as expected if the breakdown of glucose occurred according to the Embden-Meyerhof theory. The phosphoglycerate thus accumulated in human and rabbit blood was found to be the diphosphoric ester. However, the phosphoglycerate thus formed in beef blood (which normally contains no phosphoglycerate) proved to be the monophosphoric ester, indicating that monophosphoglyceric acid may be the physiologic intermediate in the glycolytic process of this blood. Recent studies reported from Warburg's laboratory may lead to a better understanding of the mechanism of formation of diphosphoglycerate in blood cells and of relationships between the mono- and diphosphoric esters. Using purified enzyme preparations from yeast, Negelein and Broemel (102) found a labile compound formed as a precursor of monophosphoglyceric acid. This substance appeared to be a diphosphoglyceric acid but differed in its constitution from the stable 2, 3-diphosphoglyceric acid of blood cells. Monophosphoglycerate was formed from the labile ester by dephos-

phorylation. These findings suggest that in blood cells which normally have a high content of diphosphoglyceric acid a labile diphosphoglycerate may be transformed into the stable isomer, whereas in other cells a dephosphorylation of the labile compound with formation of monophosphoglyceric acid takes place.

In the breakdown of diphosphoglyceric acid in blood it has been suggested that rephosphorylation of adenylic acid takes place at the expense of diphosphoglycerate (110), but details of this mechanism are unknown. Whether monophosphoglycerate or phosphopyruvate or both are intermediary products of the degradation is uncertain.

There is evidence that an unidentified phosphoric ester, comprising about 5 to 10 per cent of the organic acid-soluble P in human and rabbit blood, also takes part in glycolysis. This substance is characterized by a water soluble barium salt and is probably a carbohydrate ester. Preliminary experiments with radioactive phosphorus indicate that this fraction is rapidly rejuvenated, and other findings suggest that it may be a precursor of phosphoglyceric acid.

In nucleated erythrocytes, organic acid-soluble P compounds take part in both aerobic and anaerobic processes. Several investigators reported evidence indicating that adenosinetriphosphate in avian blood participated both in respiration and in glycolysis, but that it was maintained at its normal level only during aerobic conditions (6, 29, 30). Lyubimova (85) reported similar findings on the adenosinetriphosphate in rabbit leucocytes. Studies of Braunstein and Severin (17, 124) on avian erythrocytes suggest that adenosinetriphosphate also may be concerned with the metabolism of protein. Although avian blood normally contains no phosphoglycerate, we have found that phosphoglycerate accumulated in avian blood cells during incubation in the presence of NaF and pyruvate (unpublished work). This finding again lends further support to the validity of the Embden-Meyerhof theory of glycolysis. Phytic acid, present in large amounts in bird and turtle blood cells, appears not to take part in the glycolytic process, but undergoes a slow aerobic turnover.

Differences in concentration and distribution of phosphorus compounds in the bloods of different species may depend on either qualitative or quantitative differences in any of the numerous steps in the glycolytic process as it occurs in different bloods; also, differences in glycolytic rates may be related to differences in the normal concentration and distribution of the acid-soluble P compounds in the different bloods. Erythrocytes which are normally characterized by a low con-

centration of organic acid-soluble P, as in the ungulates, generally show a low rate of glycolysis. However, other species of erythrocytes characterized by higher concentrations of organic acid-soluble P show widely varying rates of glycolysis: human and rabbit bloods show rapid glycolysis, but pig blood with a much higher concentration of organic acid-soluble P in the erythrocytes has a low glycolytic rate. Barrenscheen and Vasarhelyi (13) suggested a rough correlation between glycolytic rate and the concentration of adenosinetriphosphate in blood cells of different species (for comments, see Kerr and Daoud, 80). Engelhardt and Ljubimowa (33) found that the rate of glycolysis in bloods of different species was correlated with the amount of organic acid-soluble P that was broken down when the blood was incubated in the absence of glucose. In addition to differences in the distribution of phosphorus compounds, however, the pH, ionic milieu, and concentrations of various coenzymes also must be considered as factors which influence the rate of glycolysis in different bloods and in different conditions. The permeability of erythrocytes to glucose has been shown by Kolotilova and Engelhardt (32, 83) to be another factor influencing the glycolytic rate in different bloods.

In experiments with blood *in vitro* Lawaczeck (84) and others (41, 93, 120) found that shifts in pH of the blood from normal toward greater acidity led to a rapid decrease in concentration of the organic acid-soluble P in the cells; this decrease was found later to be due mainly to decomposition of diphosphoglycerate (110). Roche and Roche (117) found that with increased acidity of blood the glycolytic rate diminished. Recent experiments with radioactive P (unpublished work) indicate that the rate of turnover of phosphorus in the phosphoric esters is reduced with increased acidity of the blood. Changes of pH toward greater alkalinity than normal tend to produce effects opposite to those of increased acidity. The changes in pH may be assumed to concern mainly the processes preceding the oxidation-reduction reactions which lead to the formation of phosphoglycerate and lactic acid, most likely the phosphorylation of glucose by adenosinetriphosphate. The decomposition of diphosphoglycerate with rephosphorylation of adenylic acid seems to be less affected by changes of pH.

Numerous investigators who have studied the rate of glycolysis in human blood *in vitro* have found fairly constant rates in health and widely varying rates in disease (see reviews by John, 67; Falcon-Lesses, 36; Barer, 8). Data on the rates of glycolysis and concomitant changes of inorganic P in the bloods of infants and children with various diseases

were reported by Guest (47). Rapid glycolytic rates and rapid liberation of inorganic P were observed in blood samples from patients suffering with several different conditions: most notably, gastrointestinal intoxication, and nephritis with acidosis. Thus not only glycolysis but also the dismutations of organic phosphates in the blood may be affected by conditions arising in different diseases. Such alterations of the glycolytic process may be related to disturbances in the metabolism of other tissues.

*Relation to general phosphorus metabolism.* Since the inorganic P carried in the blood is subject to esterification and subsequent liberation through reactions of the glycolytic cycle, the phosphoric esters in the blood cells may be said to participate to some extent in the general turnover of phosphorus in the body. Observations on several conditions to be discussed later in this review suggest that the phosphoric esters in the blood cells serve as carriers of phosphorus which may be utilized in tissues or may leave the body in excreta. Present knowledge makes it difficult to estimate the relative importance of this phosphorus-carrying rôle of the phosphoric esters in the blood cells, or the extent to which these esters are actually concerned in the general phosphorus metabolism of the body. There is reason to believe that the turnover of phosphorus between plasma and cells is small compared with the total transport of inorganic P in the blood. It does appear, however, that the concentration of organic acid-soluble P in the blood cells constitutes an index of the *state* of the labile phosphorus reserves of the body, inasmuch as the concentrations of the phosphoric esters in the blood cells decrease or increase as those reserves are exhausted or replenished or are actively mobilized. These relationships are discussed in later sections of this paper dealing with acidosis, nephritis, rickets, etc.

*Ionic equilibrium in the blood.* In 1923 Van Slyke, Wu and McLean (130) found the buffer value for the non-diffusible constituents of the red blood cells of a horse much higher than should be expected from their hemoglobin content, and ascribed this difference to the conjugated phosphates. Similarly, Maizels and Paterson (90) found that a considerable part of the base-binding property of the erythrocytes of patients with hypochromic anemia was due to non-diffusible acids other than hemoglobin. Guest and Andrus (48) observed a striking reciprocal relationship between decreases of chloride and increases of organic acid-soluble P in the blood cells of dogs after pyloric obstruction. Ashley and Guest (5) reported similar relationships between changes of Cl and organic phosphates in the blood cells of dogs and rabbits

after abrupt suppression of renal function. Such observations indicated that the phosphoric esters not only comprised a considerable part of the anions in normal blood cells, but that they also played an important rôle in adjustments of the acid-base equilibrium of the blood in pathologic conditions.

Increased knowledge of the nature of the organic acid-soluble phosphates now makes possible a fairly complete summation of the electrolytes in blood cells. This has been demonstrated by analyses of dog blood, before and after pyloric obstruction, and of human blood during and after recovery from the severe acidosis of diabetic coma (51, 52, 111). The anion equivalencies of the organic phosphates were calculated by means of data on the titration curves of adenosinetriphosphate, determined by Lohmann (87), and of diphosphoglycerate, determined by Kiessling (81) and by Rubin (121). The anion equivalency of diphosphoglycerate in human and dog blood cells is normally around 30 m.eq. per kgm. of water. In severe acidosis its concentration was practically 0, and following pyloric obstruction its concentration was doubled. In the reported examples of data on each of these types of blood, the sums of the determined anions in the cells (Cl, $HCO_3$, hemoglobin, and the organic phosphates) agreed closely with the concentrations of total base determined in the cells—indicating that practically all the anions in the cells had been accounted for.

As non-diffusible anions the organic acid-soluble P compounds in the blood cells appear to influence the distribution of diffusible ions between serum and cells in the manner postulated by Van Slyke, Wu and McLean (130), conforming in a general way with Donnan's law concerning the distribution of diffusible and non-diffusible ions on two sides of a semi-permeable membrane. This was demonstrated (52, 111) by calculations based on the data referred to in the preceding paragraph. Using the formulae evolved by Van Slyke, Wu and McLean (130) and taking into consideration certain correction factors determined since the date of their studies, distribution ratios were calculated from data on the non-diffusible anions and compared with observed distribution ratios of the diffusible ions $H^+$, $Cl^-$ and $HCO_3^-$. The agreement found among these ratios indicated that the distribution of the diffusible ions between the cells and serum depended mainly on the concentrations and anion equivalencies of hemoglobin and diphosphoglycerate, the two most important non-diffusible constituents of the cells.

In evaluating thus the rôle of diphosphoglycerate and hemoglobin as non-diffusible anions in the cells, it is to be emphasized that the im-

portant adjustments in anion equivalency of these two substances occur differently. In different conditions the anion equivalency of hemoglobin may change markedly with shifts in pH of the blood, but the hemoglobin content of the circulating cell does not change during short periods of acute acidosis or alkalosis. While the diphosphoglycerate also changes its anion equivalency somewhat with shifts in pH, its changes in concentration in such conditions are far more important. In this respect the diphosphoglycerate is actually more important than hemoglobin as a factor in the adjustments to changes in concentration of the diffusible electrolytes in the blood which take place in various pathologic conditions.

Changes in the distribution of acid-soluble phosphorus in the blood in disease and in experimental conditions. *Effects of acidosis.* In subjects with adequate renal function[1], the rapid development of acidosis due to many different causes is accompanied by a greatly increased excretion of phosphates in the urine. Excessive phosphaturia in human diabetes was observed around 1900 by Von Noorden, Mandel and Lusk and others and in 1907 Fitz, Alsberg and Henderson made the important observation that chloride acidosis experimentally produced in rabbits led to a greatly increased phosphaturia. Early work on this subject is reviewed by Forbes and Keith (40). Subsequently the fact that the excretion of phosphates serves to conserve base stores in the body in acidosis came to be well appreciated, but for several years little was learned concerning the nature of other adjustments which might be concerned in this physiologic mechanism. In 1924 Haldane, Wigglesworth and Kay (56) found that acidosis induced by the ingestion of ammonium chloride was associated with a greatly reduced concentration of organic acid-soluble P in the blood cells. Kay (69) found that this reduction was in the fraction not hydrolyzed by bone enzyme. In 1929 Byrom (19) found a similar reduction in concentration of organic acid-soluble P in the blood cells in diabetic acidosis, and also found these phosphoric esters in the blood cells to be restored to a normal concentration following effective insulin therapy. Similar findings in diabetic acidosis were reported by Meier and Thoenes (96). In 1937 Rapoport (107) identified as diphosphoglycerate the fraction of the organic acid-soluble P in the blood cells that decreased during ammonium chloride acidosis. This fraction

[1] Suppression of renal function from any cause, with failure of excretion of phosphates, introduces an important factor complicating the picture of phosphatemia in acidosis. See discussion in the section on nephritis.

later was found to be similarly reduced in the blood cells of patients with acute diabetic acidosis, and of infants suffering with severe acidosis due to gastroenteritis (51).

In acidotic conditions such as those just named, at least three closely related factors can be cited as probably influencing the decrease in concentration of diphosphoglycerate in the blood cells: namely, decreased pH of the blood, changes in concentration of chloride in the serum and cells, and a decreased concentration of total base in the cells. It has been pointed out (110) that if blood, incubated *in vitro*, be acidified to pH below 7.3 the diphosphoglycerate in the cells is rapidly decomposed and inorganic P is liberated—an effect on the glycolytic enzyme system which presumably is operative also *in vivo*. The exact relationship of the blood chlorides to changes in concentration of diphosphoglycerate in the cells is mainly a subject for conjecture. Since the relative distribution of Cl between serum and cells is closely correlated with the pH of the blood, an increased ratio $Cl_{cells} : Cl_{serum}$ can be considered merely an additional index of the acidotic condition (see 54). However, the striking reciprocal relationship that has been observed in a number of conditions between changes in concentration of Cl and diphosphoglycerate in the blood cells, apparently independent of the pH of the blood, suggests that the absolute concentration of Cl may itself influence the synthesis and decomposition of diphosphoglycerate. A decreased concentration of total base in the cells may be regarded as a factor influencing the concentration of diphosphoglycerate just as a decrease of total base in blood serum results perforce in a decrease of bicarbonate if the other anions are not reduced. This may be seen in diabetic acidosis, where much base is lost in the urine, and in the acidosis of gastroenteritis in infants where base is lost in diarrheal stools.

While the organic acid-soluble P compounds of the blood appear to be one source of the increased phosphaturia of acidosis, this source is relatively small compared with the total urinary phosphorus excretion. Acid-soluble P compounds of the soft tissues seem to be closely related to those of the blood and to be similarly affected by acidotic conditions. Data reported by Farquharson, Salter, Tibbets and Aub (37) on the proportions of nitrogen, calcium, and phosphorus in the urine of subjects during the rapid development of acidosis would indicate that the acid-soluble P compounds of the soft tissues were drawn upon before other stores for the mobilization of phosphorus. The decreases in concentration of the organic phosphates in the blood cells, constituting only a part of the general depletion of labile phosphorus reserves of the

body, thus appear to be an index of similar processes occurring in other tissues.

During recovery from acute severe diabetic acidosis, changes occurring in the concentration and distribution of acid-soluble P compounds in the blood appear to be closely correlated with the rate of restoration of the labile phosphorus reserves of the body to normal. This was discussed in connection with the case reported by Guest and Rapoport (51, chart 7). In the blood of that patient the restoration of the organic acid-soluble P to a normal concentration in the cells was slow compared with the restoration of the blood sugar, $CO_2$, and Cl, to normal. Insulin therapy was followed by a sharp fall in the concentration of inorganic P in both plasma and cells, and of adenosinetriphosphate in the cells, and the levels of both inorganic P and adenosinetriphosphate remained low until after the diphosphoglycerate reached a normal concentration in the blood cells (more than 4 days). After the first 24 hours practically no phosphorus was excreted in the urine during more than 7 days following symptomatic recovery of the patient. The reappearance of normal phosphaturia, after the distribution of phosphorus in the blood was restored to normal, was believed to indicate that a state of equilibrium had been reached between the demand for phosphorus in the tissues and the supply of phosphorus available.

The manifestations of acidosis, such as fatigue, dehydration, hyperpnea, etc., may be described as the result of concomitant pathologic processes in blood and tissues which mutually aggravate each other. Thus the rapid development of acidosis, due either to base deficit or to the accumulation of acids in the blood, is accompanied by losses of various electrolytes from the body, dehydration, changes in the blood such as decreased pH, shifts in Cl distribution, decomposition of diphosphoglycerate, etc. Excessive phosphaturia leading to depletion of the labile phosphorus reserves of the body may result in profound disturbances of tissue metabolism since the phosphoric esters in the tissues are concerned with many different cellular activities.

Haldane, Wigglesworth and Kay (56) stated that some of the ill effects of acidosis might be due to the depletion of the labile phosphorus reserves of the body, and suggested that in such conditions the administration of phosphate might be advantageous. We have studied the effects of the administration of phosphates in the treatment of three types of acidosis: namely, acidosis due to the ingestion of ammonium chloride (51, chart 5); diabetic acidosis (53); and acidosis due to gastroenteritis in infants (50; 51, chart 6). Although incomplete the results

suggest that in such conditions the administration of phosphates, by mouth and intravenously and in connection with other therapeutic procedures usually employed, may hasten symptomatic recovery and the restoration of a normal distribution of phosphorus in the blood.

*Alkalosis.* As pointed out in the discussion of glycolysis, reactions of the blood above pH 7.3 have been shown to favor esterification of inorganic P in the blood cells *in vitro.* Apparently no studies have been made of the influence of alkalosis alone on the distribution of organic phosphates in the blood *in vivo.* Haldane and co-workers (56) showed that alkalosis created by overbreathing resulted in a sharp fall in concentration of inorganic P in the blood (see also Holtz, 64) and diminished excretion of phosphates in the urine. It is probable, however, that increased alkalinity of the blood can produce only a small increase in the concentration of organic phosphates in the cells unless at the same time the concentration of chloride is decreased.

*Hypochloremia.* The concentration of organic acid-soluble P in the blood cells has been found to increase concomitantly with decreases in concentration of chloride in the blood cells of dogs following pyloric obstruction (4, 48), of dogs and rabbits following bilateral nephrectomy, and of dogs, rabbits and human beings in the terminal stages of nephritis (5, 48, 51, 54). In such conditions the increase of organic acid-soluble P may be practically all accounted for in the diphosphoglycerate fraction. With the hypochloremia in such conditions the blood may become more alkaline than normal (as with vomiting), or more acid (as in nephritis), or may maintain a normal pH. The importance of increases in the concentration of diphosphoglycerate in compensating for losses of Cl from the blood cells in such conditions has already been discussed.

*Nephritis.* Since much of the existing literature dealing with the phosphatemia of nephritis was reviewed by Peters and Van Slyke (105) in 1931, it seems unnecessary to repeat here their citations of this extensive literature. In their discussion of the significance of increased concentrations of inorganic P in the blood as a sign of failure of renal function, they pointed out that the inorganic P of the blood is abnormally high in cases of nephritis with gross retention of nitrogenous waste products, that the hyperphosphatemia parallels increases in blood creatinine rather closely, the blood urea less closely, and that considerable prognostic importance has been accorded to the concentration of phosphate in the blood serum in nephritis. They stated, however, that known facts did not justify an assumption that the hyperphos-

phatemia of nephritis could be due entirely to disability of the kidneys to excrete phosphates. With regard to hyperphosphatemia as a contributory cause of the acidosis of terminal nephritis, they cited evidence that while hyperphosphatemia in nephritis is usually accompanied by lowered plasma bicarbonate and acidosis, the increased phosphoric acid in the blood plays a less important part in producing acidosis than other combined acidogenic factors.

Present knowledge makes it difficult to estimate the relative importance of "retention" *per se* as a factor influencing the level of inorganic P in the blood in nephritis and other conditions attended with suppression of renal function. In such conditions, however, it appears that increased concentrations of both inorganic and organic phosphates in the blood are due to the combined effects, direct and indirect, of renal damage and of various extrarenal factors such as those mentioned in the preceding discussion of ionic equilibrium and glycolytic processes in the blood. The renal and extrarenal factors respectively may have more or less influence upon phosphatemia according to the type and severity and stage of the disease. In any case, the organic as well as the inorganic phosphates in the blood should be considered when evaluating the significance of hyperphosphatemia in nephritic acidosis.

Increased concentrations of organic acid-soluble P in the blood cells of patients suffering with nephritis and other renal disorders were reported by Byrom and Kay (20) in 1927. In such bloods they found the fraction of the ester P hydrolyzable by bone enzyme increased, and the non-hydrolyzable fraction decreased. Hoesch (63) reported somewhat similar data on the bloods of nephritic patients, showing the fractions of the organic acid-soluble P hydrolyzable and non-hydrolyzable by N HCl to be increased and decreased, respectively. Stearns and Warweg (129b) reported increases of organic acid-soluble P in the blood cells of children suffering with severe renal damage. In 1934 Ashley and Guest (5) found that the acute suppression of renal function that resulted from injections of diphtheria toxin and $HgCl_2$, and bilateral nephrectomy, led to increases of inorganic P in the blood plasma and cells and of organic acid-soluble P in the blood cells, accompanied by decreases of chloride. The increases in concentration of organic acid-soluble P in the cells occurring in such conditions later were accounted for by increases in the diphosphoglycerate fraction (51, 54).

Some of the effects of abrupt cessation of renal function are illustrated in chart 3 of Guest and Rapoport (51), representing changes found in the blood of a dog during 7 days following bilateral nephrectomy.

Those changes were: 1, progressive increase of non-protein nitrogen; 2, increase of inorganic P, somewhat slower at first than later; 3, increase of organic acid-soluble P in the cells, practically all accounted for by nearly equal increases in the diphosphoglycerate fraction; 4, decrease of $HCO_3$ and Cl in both serum and cells. The changes found in the blood samples drawn the last two days of this experiment correspond closely with those we have found repeatedly in blood samples drawn from patients in the terminal stages of chronic nephritis when renal function was reduced nearly to nil. A much more varied picture may be drawn, however, of changes which occur in the bloods of patients in different stages of acute and chronic nephritis. In table IV of a recent paper (54) are listed data on 4 blood samples from patients suffering with chronic nephritis with varying degrees of renal impairment and in different conditions of acidosis. In such patients normal, low, or high concentrations of organic acid-soluble P in the blood cells may accompany decreases of $HCO_3$ and pH of the serum. Such findings suggest that the phosphatemia of nephritis is subject to a balance between the effects of acidosis and phosphate retention, respectively favoring decreases and increases of organic acid-soluble P in the cells. Thus acidosis from any cause in a nephritic patient may result in a decreased concentration of phosphoric esters in the blood cells if the excretory function is adequate. If, however, phosphate excretion fails, the concentration of organic acid-soluble P in the cells may increase in spite of a lowered pH of the blood plasma. During periods of rapid change in the general condition of nephritic patients due to acute infections or other causes, we have occasionally observed large fluctuations in the relative proportions of adenosinetriphosphate and diphosphoglycerate in the blood, finding concentrations of adenosinetriphosphate as high as 30 mgm. per 100 cc. of cells. Such observations are in accord with the findings of Byrom and Kay (20), and of Hoesch (63), that the hydrolyzable fraction of the organic acid-soluble P in blood cells of nephritic patients was relatively increased, compared with the non-hydrolyzable fraction.

As has been described in other conditions, changes in concentration of various electrolytes in both plasma and cells occur concomitantly with changes in the distribution of phosphorus in nephritic bloods. Therefore the concentrations and distribution in serum and cells of the Cl, total base, etc., must be included in the consideration of factors which may influence the changes in distribution of phosphorus in the blood in any given case of nephritis.

*Rickets.* Although most studies of phosphatemia in rickets have been limited to the inorganic P of plasma or serum, there exists much evidence that changes in the concentration of organic acid-soluble P compounds of the blood cells are important in the chemical pathology of this condition. Iverson and Lenstrup in 1920 (65) and later Freudenberg (41) found an increase of total acid-soluble P in the whole blood of rachitic infants during the period of healing of rickets, and in 1925 Robison and Soames (116) found in the whole blood of rachitic rats a reduced concentration of phosphoric esters hydrolyzable by bone enzyme (see also Euler and Brandt, 35). Wilder (137) found increases of organic acid-soluble P in the blood cells of rachitic rats during the healing that occurred with fasting. A reduced concentration of readily hydrolyzable organic phosphate in the blood cells of rachitic infants was reported by Bakwin, Bodansky and Turner (7a) and similar findings on the blood of rachitic rats were reported by Jacobsen (66). Bakwin and co-workers later (7b) found the lowered concentration of organic acid-soluble P in the blood cells of rachitic infants to be partly accounted for by decreases in the readily hydrolyzable fraction, adenosinetriphosphate, and partly by decreases in the fraction not hydrolyzed by bone enzyme—a fraction they surmised would prove to be diphosphoglycerate. Stearns and Warweg (129a) found increases of ester P in the blood cells of three children during the healing stages of late rickets. Kay (72) and Skill and Kay (126) reported that various types of experimental rickets (including "Beryllium rickets") were associated with a great reduction in the concentration of phosphoric esters in the blood cells and in the liver as well as with decreased concentrations of inorganic P in the blood. Kay and Skill (76) reported also that recovery of rachitic rats was associated with a rapid increase in the phosphoric ester content of the blood cells. Kay's observations of parallel decreases of organic acid-soluble P in blood cells and in liver may be cited as important evidence that changes in the concentration of phosphoric esters in the blood cells constitute an index of the state of the labile phosphorus reserves of the whole body.

We have reported data (51, 109) showing that the development of rickets induced in rats by a high Ca low P diet was associated with decreases first of inorganic P and of adenosinetriphosphate and then of diphosphoglycerate in the blood cells. During the development of rickets the progressive decrease in concentration of diphosphoglycerate in the blood cells appeared to reflect an increasing deficiency of phosphorus in the rats. Healing of the rickets, induced by a minimum

healing dose of vitamin D, or by the addition of phosphate to the diet, or by fasting, was preceded by increases first of diphosphoglycerate and then of adenosinetriphosphate and inorganic P in the blood cells. A rapid increase in concentration of diphosphoglycerate in the blood cells following any of the procedures which induced healing appeared to be a sign of rapid mobilization of phosphorus in the body and of the availability of such phosphorus for transport to the calcifying bones.

Also of interest in this discussion of rickets are some unpublished observations we have made on the distribution of phosphorus in the bloods of monkeys in relation to intake of vitamin D and seasonal exposure to sunlight. A monkey that had been kept in the laboratory several years had developed great weakness—apparently the condition commonly designated "cage paralysis." The concentration of inorganic P in the blood was 1.1 mgm. per 100 cc., and that of organic acid-soluble P in the cells, 28 mgm. per 100 cc. Roentgenograms of the bones disclosed extreme osteoporosis. Vitamin D therapy without other change in the diet was followed by a rapid improvement in the general condition of the monkey and restoration of the blood phosphorus to normal levels. When we subsequently examined a number of apparently normal monkeys that had been kept in the laboratory one to two years, several were found to have low concentrations of both inorganic and organic phosphates in the blood and most of these had definite roentgenographic signs of rickets or osteoporosis. Low concentrations of inorganic and organic phosphates also were found in the bloods of supposedly normal young monkeys newly arrived in the laboratory during winter months, although roentgenographic studies of their bones not always revealed visible signs of rickets. The administration of vitamin D to these monkeys was followed by increases of the blood phosphorus to normal concentrations. Monkeys of the same laboratory kept out of doors during summer months were found to have normal concentrations of inorganic and organic phosphates in their bloods.

*Overdosage effects of irradiated ergosterol.* In 1933 Guest and Warkany (55) reported that the administration of large doses of irradiated ergosterol to normal rabbits was followed by large increases in the concentration of organic acid-soluble P in the blood cells. Later, we found these changes to be practically limited to the diphosphoglycerate fraction (49). Observations made in the course of the latter studies suggested that the changes in concentration of organic phosphates in the blood cells were secondary to effects of the irradiated ergosterol

on other body tissues. In exsanguination-replacement blood transfusion experiments, blood was exchanged between normal rabbits and rabbits intoxicated with high doses of irradiated ergosterol. In blood cells from normal rabbits, injected into intoxicated rabbits, the concentration of diphosphoglycerate quickly increased to a level previously determined in the intoxicated rabbit's own blood cells. In blood cells from intoxicated rabbits, injected into normal rabbits, the concentration of diphosphoglycerate fell quickly to the normal level previously determined in the recipient's own cells. That the diphosphoglycerate in the blood cells thus proved to be so labile *in vivo* under such conditions is another indication that its concentration is governed largely by the state of the labile phosphorus reserves of the body.

Because of these experimental observations, it was suggested (25) that changes in the distribution of phosphorus in the blood might afford a sign of approaching intoxication in patients treated with large doses of vitamin D for various reasons. Studies were subsequently made on the bloods of several adult arthritic patients who were given daily doses of 50,000 to 500,000 international units of vitamin D for varying periods of time. However, only slight changes were found in the distribution of acid-soluble P in the blood cells of these patients even when the titre of vitamin D in the blood serum rose to as high as 13,000 units per 100 cc. (132). No attempt was made to increase the daily doses of vitamin D given these patients to amounts causing more than mild symptoms of intoxication.

*Parathyroid hormone.* Various investigators studying relationships between the parathyroid glands and phosphorus metabolism have found that injections of parathyroid extract are followed within a few hours by a greatly increased excretion of phosphorus in the urine, with a concomitant decrease in the concentration of inorganic P in the blood (2, 42). These changes preceded the effects of this hormone on calcium metabolism—increased calcium excretion and increased concentration of calcium in the blood—effects which usually were not apparent earlier than 24 hours after the injection. Cantarow, Brundage and Housel (22) reported data on changes of organic acid-soluble P in the blood of dogs following injections of large doses of parathyroid extract showing that the organic acid-soluble P in the blood cells first decreased and later, with the development of dehydration and failure of renal function, increased. We have confirmed these observations and found that changes in the diphosphoglycerate accounted for the changes of organic acid-soluble P. Thus it appears that the first effect of large doses of

the parathyroid hormone is a mobilization of labile phosphorus reserves. With a decreased renal threshold for phosphate excretion (1, 28, 58), increased phosphaturia leads to a decrease in the concentration of organic acid-soluble P in the blood cells. Subsequently, the development of dehydration and failure of renal excretion (125) probably account for the increases of inorganic and organic acid-soluble phosphates in the blood.

*Anemias.* Byrom and Kay (21) found a high concentration of organic acid-soluble P in the blood cells of patients with pernicious anemia and leukemia. Warweg and Stearns (133) studied the distribution of phosphorus in the blood of a patient during recovery from a severe anemia caused by prolonged gastro-intestinal hemorrhages. During the initial period of recovery the organic acid-soluble P in the blood cells increased to a high concentration and then decreased gradually as the cell count and hemoglobin content of the blood approached normal. Kinzel (82) reported a decrease of organic acid-soluble P in the blood cells of rabbits subjected to bleeding and treatment with phenylhydrazine—results which are not in accord with the findings of other investigators who made similar experiments. Malan (92) reported increased concentrations of organic acid-soluble P, with the appearance of considerable amounts of nucleoprotein, in the blood cells of anemic sheep and cattle. In newborn infants with severe jaundice, anemia, and erythroblastosis neonatorum, we have observed rapid increases in the concentration of organic acid-soluble P in the blood cells occurring during the first few days of life. Following recovery from these conditions, the organic acid-soluble P decreased to a normal concentration in the cells within a few days.

## SUMMARY

In this review attention is directed mainly to available information concerning the functions of the organic acid-soluble phosphorus compounds in mammalian bloods, and to changes in the concentration of these compounds in the blood observed in different conditions.

The distribution of the phosphoric esters in normal blood varies greatly in different species, and among normal individuals of a species the distribution may vary according to age, type of diet, and other factors.

As non-diffusible anions in the cells, these compounds are important factors in the electrolyte equilibrium of the blood. Inasmuch as the phosphoric esters are synthesized and decomposed through reactions

of the glycolytic cycle, the enzymatic processes of glycolysis have an important dynamic rôle in the maintenance of the electrolyte equilibrium of the blood.

The greatest changes in concentration of organic acid-soluble phosphorus in the blood cells in pathologic conditions are usually in the diphosphoglycerate fraction, at least in human, dog, rabbit, and rat bloods, the species most studied. This fraction is decreased with phosphorus deficiency in rickets and in various types of acidosis in subjects with adequate renal function, and increased after pyloric obstruction, after suppression of renal function, and in some other conditions. The significance of the changes in distribution of phosphorus in the blood in such conditions is discussed with regard to various metabolic disturbances, and to the state of the labile phosphorus reserves in the body.

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# THE NUTRITION OF THE FETUS

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Numerous factors influence the healthy development of the embryo. They include the maternal supply of foodstuffs adequate to the fetal requirements both in regard to quantity and quality, the sources from which they are derived and their utilisation in the peculiar metabolism of growth and finally their modifications by species, heredity and disease.

The assessment of the state of fetal nutrition is difficult; pre-natal estimations of nutritional condition are difficult to make so long as the fetus is in the uterus. Obstetrical and radiological examinations and the assessment of maternal health are the only methods available, but their results are limited and relatively crude.

The post-natal indices include birth weight, vigour and post-natal growth, and of these the birth weight is probably the best at our disposal in the absence of obvious disease. In animals the greater the size at birth the better the nutrition but while this may be so in the human the complication of the relatively greater size of the human fetal cranium presents obstetric difficulties not encountered in animals. Strength and development of muscle is impossible to assess quantitatively in the newborn as are differences of weight due to bone, muscle and fat. Since, however, muscle and bone are the heaviest tissues, these if well developed conduce to a higher weight at birth. Although the birth weight is only a rough measure of nutritional state it does appear to give an indication of the future post-natal development and there is ample evidence to show that the rate of growth after birth is a measure of the condition before or at birth.

Before considering the metabolic development in the uterus it is necessary to outline briefly the comparative physiology of the fetus since most of the work has been performed in animals and it is not always possible to argue from one species to another, still less to man.

*Comparative physiology.* The Mammalia are divided into three main nutritional groups, the Monotremata (Echidna and Ornithorhynchus),

the Marsupialia and all other members of the Eutheria. In the first group an egg is laid, the yolk of which supplies the earliest nourishment (telolecithal). After laying it is carried on the maternal abdomen and the young, when hatched, feeds at its mother's nipples. In the Marsupialia, the young are born immature and are carried at the maternal hypogastric nipples. The ovum before birth is alecithal and the foodstuffs are derived by a yolk sac placenta. In the remaining Eutheria the foodstuffs are derived first from the residues of the discus proligerus and zona radiata (prochorion), later from the yolk sac placenta and finally by the allantoic vessels from either an allantoic placenta or a chorionic placenta.

The yolk sac varies with the species. It is large in the Rodentia, smaller in the Ungulata especially in comparison with the allantois. In the Insectivora its vessels penetrate the trophoblast but in the anthropoid apes and Man it is small and never leaving the body it never reaches the trophoblast (extra-embryonic ectoderm).

The allantois is the main source of nourishment in the Eutheria. Its mesodermal sac grows out from the entodermal hind gut in all mammals except Tarsius and the Primata where it merely forms a small outgrowth into the body stalk. The Primata have a chorionic placenta, that is, there is no allantoic sac entering it, but the allantoic (later called the umbilical) vessels grow into the mesodermal body stalk to the chorion composed of mesoderm and ectoderm (trophoblast) and thrown up into chorionic villi with a core of vessels within the villus.

The fetal portion of the placenta is derived from the trophoblast and the maternal part from the decidua. In the fully formed placenta there are six main tissues intervening between the fetal blood and the maternal blood, namely, the endothelium of the maternal blood vessels, the connective tissue or stroma of the decidua containing glands, the epithelium covering this and apposed to the fetal chorion, the chorion itself derived from the trophoblast, the connective tissue of the fetal villi and lastly the endothelium of the fetal vessels. The most characteristic fact about the placenta in all species is that it always undergoes degenerative changes toward the end of uterine life. In consequence there are hemorrhages of the maternal blood, which may stagnate and autolyse; the fetal chorionic villi appear to absorb as foodstuff the glandular decidual secretion, the autolysed uterine tissue and hemorrhages. This necrosis progresses to different stages in different species. Grosser (1925) classifies the placenta according to the degree of degen-

eration and his classification is of most use in considering the layers across which foodstuffs have to transfuse. They are named after the maternal tissue apposed to the fetal tissue. There are four main types:

1. Epithelio-chorial. No necrosis. Decidual epithelium apposed to chorion: in the pig and horse.
2. Syndesmo-chorial. Decidual epithelium disappeared; seen in ruminants.
3. Endothelio-chorial. Gross degeneration and hemorrhages of the maternal connective tissue; in carnivora.
4. Hemo-chorial. Chorionic villi immersed in maternal blood; in rodents, apes and the human.

Mossman (1926) states the fact that in the rat and the rabbit the chorion may degenerate yielding only the fetal endothelium between the two bloods (hemo-endothelial). In the hemo-chorial types the blood does not stagnate and autolyse but circulates as in the splenic sinuses.

*The embryological foodstuffs.* This term is used to cover those materials in the ovum or uterus which do not come directly from the maternal diet. It includes:

1. The yolk of the egg in the Monotremata. Histologically it resembles that of the Sauropsida but there do not appear to be any recent analyses available.
2. The milk of the Marsupialia and the Monotremata. Wood Jones (1939) provides an analysis of the milk of the monotreme echidna. The outstanding fact is the very high percentage of solids and of the fat content.

| | |
|---|---|
| Specific gravity | 1.023 |
| Total solids | 36.75 per cent |
| Casein (?) (pH 4.7) | 8.4 per cent |
| Albumen and globulin | 2.9 per cent |
| Undetermined nitrogen | 0.34 per cent |
| Lactose | 2.81 per cent |
| Fat (Sapon. value 0.192) | 19.62 per cent |
| Pigment | Little or none |
| Ash | 0.78 per cent |

3. The prochorion.
4. The embryotrophe. This includes the secretion of the uterine glands, and also the necrotic residues derived from the decidua and maternal hemorrhages. It is of greatest importance in the Ungulata. Analysis was made by Gamgee (1864).

The principal components are:

1. Cellular elements—blood cells and desquamate decidua.
2. Fluid elements—blood, lymph and glandular secretion.

The chief chemical compounds are proteins (10 per cent); glycogen and traces of fat (mainly intracellular); pigments which include hemoglobin and uteroverdin, a green derivative of hemoglobin peculiar to the carnivorous placenta; salts and crystals especially calcium oxalate. The phagocytic trophoblast cells ingest these compounds so that they are visible in the chorionic villi. The evidence for the chemistry of the embryotrophe is almost entirely histological.

*The increase in fetal weight.* The rates of deposition differ for different substances and at different ages. More than half the weight is deposited in the last six to eight weeks when there is accelerated deposition of all materials. For this reason a premature infant will put on weight more rapidly than a full term child. Water deposition is pronounced but that of solids is still more rapid, so that the water content of the body drops from 96 per cent at the end of the 2nd month to 74.5 per cent at term. Considering the dry weight, Needham has shown analogies with the bird in that the solid materials are neither deposited regularly during fetal life nor do they increase in quantity with a crescendo effect in the last two months but rather does one find variations in the percentage deposition signifying different rates of anabolism at different ages. The ash as a percentage of the dry weight rises till the 4th month (human fetus) and then remains constant, the fat shows a steady rise in the last quarter, whereas the protein has a peak at the 7th month and actually decreases in the subsequent period (as a percentage of the dry weight).

The daily demands of the fetus are shown in table 1.

The source of these materials may be the maternal tissues or the maternal diet. The relative utilisation of these two sources is not known nor is it possible to state in what proportion the mother's food goes to her own basal endogenous metabolism, to new tissue formation in her body or to fetal tissue formation.

Analyses have been made of the relation between fetal age and fetal growth by a number of workers using mathematical methods (Minot, 1891, 1908; Robertson, 1923; McDowall, Gates and McDowall, 1930; Brody, 1928; Schmalhausen, 1928). Their varying methods (logarithmic plotting, differentiated analysis, etc.) have yielded interesting results, the most important being some success in demonstrating that rate of growth can depend on the velocity of the catalysing reactions

concerned and that at different ages the changes in growth rate are controlled by new reactions with different velocities. The data available do not however permit any sweeping conclusion; it is not possible to consider the problem in the limits of this review but full discussions can be found in Needham (p. 368) and in Brody (1928).

*The birth weight.* Rubner (1908) first showed that in all species except man, the birth weight is proportional to the gestation time. In man it is below the expected weight. The reason for this is unknown, though it is as well to point out that the human has a disproportionately big head, especially in relation to the pelvic outlet.

Apart from this the most important factor is the food supply of the mother. Maternal overfeeding appears to have no effect on the birth weight but to increase the maternal weight in the sheep (Hammond, 1933). Moderate underfeeding has no effect in gilts (Hogan, 1928) nor in the sheep where however it depletes the maternal reserves and results in loss of weight (Hammond, 1933); and in the cow Eckles (1919) has found the same effect and regards breed as of greater importance. The same may be the explanation of the observations on birth weights in Central Europe in the 1914–19 War. On the other hand both Prochovnik, (1899 and 1901), and Jonen (1932) claim that restricted diet lowers the human birth weight, as also do Acosta-Sison and Calang (1931) in the Philippine Islands. In rats (Zuntz, 1924), guinea pigs (Paton, 1903) and rabbits (Reeb, 1905) underfeeding in pregnancy gives low birth weights, and Hammond (1933) has shown in sheep that the birth weight is markedly lowered if the underfeeding is so pronounced as to markedly depress the maternal plane of nutrition. Hammond's results as to moderate feeding in sheep depleting the maternal

TABLE 1

*Composition of the human fetus in grams*

| | BODY | WATER | PROTEIN | FAT | ASH |
|---|---|---|---|---|---|
| End of 5th lunar month | 300 | 260 | 22 | 3.5 | 1.5 |
| End of 7th lunar month | 1,000 | 800 | 100 | 25.0 | 30 |
| End of 10th lunar month (birth) | 3,200 | 2,420 | 400 | 350.0 | 90 |
| *Average of daily deposition:* | | | | | |
| 1. Throughout fetal life | 11.4 | 8.6 | 1.4 | 1.25 | 0.32 |
| 2. In last 3 lunar months | 26.2 | 19.3 | 3.57 | 3.87 | 0.71 |
| 3. In last month | 35.7 | 23.6 | 6.4 | 5.0 | 2.0 |

reserves are confirmed in the human where it is known both loss of weight and loss of specific materials such as calcium, phosphorus, iron and vitamin D can occur with shortage of these or of calories or protein in the diet (see the later sections of this review and also Garry and Stiven, 1936).

The influence of the maternal food supply on the fetal development and birth weight is modified by competing metabolisms, notably the mother's own metabolism and that of twins, triplets or a litter. Hammond (1933) showed that the birth weight of single lambs was 29 per cent heavier, and that of individual triplets 9 per cent lighter than that of members of the twin pairs. The size of young at birth is inversely proportional to the number in litters of pigs, guinea pigs and rabbits. In addition if the ewe before lambing be ill-nourished then the birth weight of individual twins and triplets are less and the difference between their weights and that of single lambs at birth is proportional to the degree of malnutrition of the mother. Put differently the ratio

$$\frac{\text{Weight of single lambs}}{\text{Weight of individual twin lambs}}$$

was proportional to the nutritive impairment. This ratio is normally greater for female lambs than for male lambs from which he infers rams have a better chance of survival than ewe lambs and that in those species where the ratio is small there is a better chance of survival than in other species. There is no direct evidence of how this comes about or of the effects of male or female sex hormones on the growth of lambs in the uterus.

Hammond (1933) has also shown in the sheep that the birth weight is dependent on the ewe weight at birth. This in turn depends on 1, her nutritive condition: if deficient she is competing with her fetus; 2, her age: if still growing she is competing again with her young; 3, the species. He showed that the diet in the last weeks of pregnancy influenced the birth weight, summer lambs being heavier than early spring lambs. Toverud (1933) has shown in humans that the infant birth weight is highest in August and lowest in January. Vitamin A concentrates administered to pregnant cattle have been shown to increase the birth weight of the calves (Massachusetts 1938). The age of the ewe influences her birth weights in that her lambs are heavier the later the pregnancy. The same fact is well known in connection with child marriages where the birth weights (e.g., in India) are less than with adult marriages.

*Carbohydrate metabolism in the placenta and fetus.* Claude Bernard (1859) showed that rabbit's blood sugar was fructose and that it was also present in the amniotic and allantoic fluids. Since then it has been shown that some species have glucose and not fructose: for example, the goat amniotic sugar is glucose (Huggett, 1929). Dickens and Greville (1932) (Warburg technique) showed that all embryonic tissues anaerobically converted glucose to lactic acid but only some formed the acid from fructose. Of these only the outer membranes of the rat and guinea pig and allantois of the rabbit attacked fructose and glucose equally. The placentae of the rabbit, guinea pig, rat and mouse had hardly any effect on fructose: the same applied to the tissues of the embryo proper: human full-time placenta was less active against fructose than against glucose and embryo tissues (rat and rabbit) had no action on fructose even though it was present in the fetal blood. The whole problem of fructose in the fetus is curious and unsolved. This work was the logical extension of the work of Warburg and his school on glycolysis under aerobic and anaerobic conditions. They showed that embryonic rat tissues only caused glycolysis under anaerobic conditions, practically not at all aerobically and never in vivo, indicating that its respiration is adequate to prevent glycolysis (distinction from the rapid growing tumour) (Warburg, 1930; Negelein, 1925).

The fetal blood sugar is very constant at any one age but rises towards 100 mgm. at full term (Snyder and Hoskins, 1928). In twins it is in each less than the maternal blood sugar (Slemons, 1919; Lawrence, 1929). Lawrence (1940) also notes a case where the maternal B.S. was 180 mgm., the umbilical vein sugar 125 mgm. and umbilical artery sugar 75 mgm. Equilibration with the maternal sugar does not occur in the placenta but there is free uptake by the fetal tissues. Ketteringham and Austin (1939) gave sugar to the mother intravenously in the second stage of labour. A rise in the sugar of both maternal and fetal bloods occurred returning to normal in four hours in the mother and in two hours in the fetus, suggesting active tissue-utilisation.

The placenta contains glycogen (Bernard, 1859) which appears at the 16th day in the rabbit (Pflüger, 1904), reaches a maximum at the 22nd day and then disappears toward term, coincident with a corresponding rise in fetal liver glycogen (Lochhead and Cramer, 1908). In man the peak is at the second month (Eufinger, 1932). Lochhead and Cramer (1908) showed it was difficult to alter the concentration of the placental glycogen experimentally. Huggett (1929) confirmed this and demonstrated it was only diminished by gross toxic katabolic

changes in the mother and that it was independent of the maternal blood sugar fluctuations. It appears to be a store of glycogen ear-marked for the fetus, within the decidual tissue. Corey (1935) confirmed this in the rat. Endeavours have been made to study its metabolism by three main techniques: 1, autolysis of placental breis; 2, placental transfusion with sugars; 3, the Warburg technique.

The subject is reviewed by Needham (1931) but it is by no means in a satisfactory position because of difficulty of technique, differences in results obtained by different observers, differences in interpretation of results which do not always appear to be entirely objective. The following points appear to be established:

1. The placenta contains an amylase capable of converting glycogen to glucose (Lochhead and Cramer, 1908; Maeda, 1923).

2. The glycogen in aerobic autolysis of the placenta yields glucose (Davy and Huggett, 1934).

3. There is practically no production in vivo of lactic acid by either the placenta or embryo as shown by lactic acid estimations in the arterial and venous bloods of the uterine and umbilical vessels (Wind and von Oettingen, 1928).

4. Autoglycolysis of the embryo or placenta forms lactic acid anaerobically (Dickens and Greville, 1932).

5. All embryonic and placental tissues anaerobically form lactic acid from added glucose but only the placenta of man, the cat and the ferret and certain fetal membranes anaerobically form lactic acid from added fructose (Dickens and Greville, 1932).

There is also the possibility that perfusion of the placenta with glucose may form glycogen (Felix and von Oettingen, 1924).

The fetal liver glycogen in contrast to the placental glycogen is much more mobile, increasing in concentration with age in the rabbit (Lochhead and Cramer, 1908), the rat (Szendi, 1936) and man (Eufinger, 1932) and with fluctuations in the maternal blood sugar (Stuart and Higgins, 1935). The latter observers find it rises and falls more rapidly than the maternal liver glycogen. The observations of Lawrence (1940) and Ketteringham and Austin (1939) have been described above. Aron (1924) suggests the rise in fetal liver glycogen coincides with the development of the pancreas. Lund (1932), by special technique, has recorded that embryonic glycogen has a specific rotatory power less than adult liver glycogen and says it is specifically different.

Szendi (1936) says the lungs are relatively rich in glycogen in early

fetal life, before hepatic activity is maximal. The metabolism of glycogen in the fetal muscles is practically unstudied; Szendi regards it as a separate store.

Insulin (in regard to carbohydrate metabolism) appears not to pass across the placenta (Schlossmann, 1931; Snyder and Hoskins, 1928) either way. The ungulate fetus is relatively resistant to insulin compared with other types, and fetal lamb plasma if injected into the fetal rabbit confers resistance to insulin (Passmore and Schlossmann, 1938). Four hundred and fifteen units per kilogram gave no fall in blood sugar in the sheep but caused a big rise in blood lactic acid. The subject is reviewed by Schlossmann (1932a).

*Lipin metabolism in placenta and fetus.* Fat has been known to exist in the placenta for some time, the evidence for its presence being mainly histological. Watanabe (1923) showed that all the ether soluble materials in the human placenta increased up to the 4th month after which there was a progressive decrease till term. Kreidl and Donath (1910) had looked unsuccessfully for a lipase in the guinea-pig placenta which however was found in the human placenta by Anselmino and Hoffmann (1929) and also was found to have its maximum activity at the 4th month, possibly a coincidence with Watanabe's date given above. Hoffbauer (1903, 1905) and later Gage and Gage (1909) both showed that stained fat administered to the mother traversed the placenta but the dye was inhibited from passing and they inferred that the passage of fat was not by diffusion alone. Slemons (1919) and also Slemons and Stander (1923) showed fetal blood fat was uniformly less than the maternal blood fat. Oshima (1906) had shown this difference for the chylomicrons also and that the fetal chylomicrons (identified conclusively with fat particles by Elkes, Frazer and Stewart only in 1939) were unaffected by an increase in natural dietary fat sufficient to raise the maternal blood fat. Ahlfeld (1877) had obtained a similar result by giving bacon fat to the pig. Several observers showed that special types of fat traversed the placenta unaltered. These included Bickenbach and Rupp (1931) (fed labelled fat and cod liver oil to rabbit; detected unaltered in the fetal blood), Sinclair (1933) (cod liver oil in rats), McConnell and Sinclair (1937) (trielaidin in the rat appearing in the phospholipins of the fetal liver and fat deposits of the embryo). Sinclair (1933) concluded that there was no evidence in the rodent (whose placental membrane most resembles the human) of how the fats passed through the placenta, whether with hydrolysis or not. Needham (p. 1479) had pointed out that despite the lipase there was no evidence of forma-

tion of glycerol and fatty acids or of fat resynthesis in the placenta. Boyd (1935) estimated the neutral fat, phospholipin, free cholesterol and cholesterol esters in the placenta and fetus (rabbit) and concluded that increased phospholipin in the fetus was associated with great activity of the placenta and this was due to active and not passive transfer of the lipoids from mother to fetus. Boyd and Wilson (1935) showed that the human fetus at birth absorbs 30 grams of phospholipin and 7 grams of free cholesterol in 24 hours and that even if the umbilical cord were clamped the fat in the umbilical vein on the placental side increased showing, they inferred, active transfer of fat by the placenta. Sinclair (1933) had estimated iodine values in fetus and food fat. Thiemich (1905) had given coconut oil (I.V. 8.) to dogs in 1905 and showed that while it transfused it did not lower the fetal depot fat I.V. but did lower that of the maternal depots. Wesson (1926) found a similar result with the bromine value in rats and inferred the fetus synthesised fat from carbohydrate. Miura (1937), however, contradicted these results and confirmed Sinclair's iodine values, using rats and giving linseed oil, coconut oil and elaidic acid, which last appeared in the fetal phosphatids. Effkemann (1936) with infants at birth showed they absorb in 24 hours 33 grams of lecithin, 7.5 grams free cholesterol (essentially the same as Boyd and Wilson), 3 grams of fatty acid and 8.5 grams of cholesterol esters. The cholesterol esters are mainly carried by the serum, the free cholesterol and phospholipin by the cells. He inferred that the maternal diet can alter the fetal depot fat, and that preformed fat compounds are used by the fetus. Chaikoff and Robinson (1933) have shown that pregnant rats on a low fat diet (with or without an excess of carbohydrate or protein) have fetal fat stores of 72 to 74 iodine value. But if the dietary fat was much increased then the fetal I.V. ranged from 61 to 103 (maternal depot I.V. 36–145) and corresponded to the I.V. of the dietary fat. In other words, carbohydrate can be synthesised to fat of I.V. 72–74 in the fetus but this value can be altered if excess fat is given. Imrie and Graham (1920) studied the guinea-pig fetal liver fat. They found that its concentration rose steadily during intrauterine life giving pronounced fatty infiltration but there was a sudden sharp drop within 40 hours of birth (suggesting an acute birth change in metabolism). Its iodine value fell steadily from 120 to 110 but was always above the adult tissue I.V. of 85 (adult liver I.V. equals 120). These high values (120–110) suggested the fetal liver fat came directly from the adult liver unaltered or that the fetal liver had high desaturation power. They now

starved the pregnant guinea pig and gave phlorhizin. This produced double the fatty infiltration in the fetal liver but with fat of a low iodine value. This fresh fat they inferred as coming from the tissues or being synthesised from carbohydrate.

Some work has been performed on cholestérol metabolism. Boyd (1935) estimated the cholesterol in the placenta and fetus of the rabbit and showed the free sterol increased steadily in the placenta but in the fetus it rose first rapidly, then slowly and again steeply in the last ten days; cholesterol esters however rose in the first half and fell in the second half of pregnancy. With Wilson (1935) he estimated the human free cholesterol absorption per day, results confirmed later by Effkemann (1936). Chauffard, Laroche and Grigaut (1918, 1920) showed the human fetal suprarenal had a steadily rising percentage and absolute weight of free cholesterol with age; Sato (1937) demonstrated that it rises in the maternal blood and falls in the umbilical artery and vein throughout pregnancy and believed the fetus synthesised it. Rosenbloom (1935) with the full term infant estimated 223 mgm. cholesterol in the mother's blood and 120 mgm. in the umbilical blood and was undecided between fetal synthesising or fetal storage with progressive age.

*Protein metabolism in placenta and fetus.* Unlike carbohydrate there are no obvious protein stores, but in the last part of pregnancy the placenta gradually wastes. The products of the autolysis are unknown nor is there any knowledge of their fate. They are, however, formed at the time of maximal fetal protein synthesis and form an obvious foodstuff for the fetus.

Legrand (1936) states there are polypeptides in the maternal blood which in pregnancy are replaced by smaller molecule peptides which can diffuse through the placenta to be utilised by the fetus. This gives some support to the older view that fetal protein is synthesised from the albumoses. The results need confirmation however by an alternative method.

The circulating amino-acid content (whole blood and serum) is invariably higher in the fetus than in the mother. The data are reviewed by Slemons (1919), Needham (p. 1516) and Schlossmann (1932a, b). Despite these figures Schlossmann argues that amino-acids diffuse from the mother to the fetus. He states the fetal level is maintained artificially high by adsorption on to the red cells and colloids, quoting Plass and Matthew (1925) in support. It is doubtful, however, if the problem is so simple of solution. Luck and Engle (1929) showed that

the experimental injection of amino-acid into the mother speedily raises the fetal tissue content.

Protein deposition is maximal in the last quarter of pregnancy. There appears to be little relation between the composition of the proteins deposited in the fetus and that of the maternal proteins. The fetal creatine rises steadily throughout pregnancy (Beker, 1913; Hunter, 1922). On the other hand the nucleo-protein decreases with intrauterine age (Masing, 1911). Goldstein and Milgrom (1935) state cathepsin catalyses the protein deposition. It exists in two forms, activated and unactivated and the synthetic action is proportional to the excess of the former over the latter. They demonstrated that synthesis occurs at a slow rate in the human placenta but faster in rodents. They found protein synthesis both in the fetus and in the maternal tissues—not a wholly unexpected finding.

Slonaker (1938) has recently fed six generations of rats on diets containing constant protein rations between 10 and 26 per cent (man normally eats 13 per cent). He found high protein decreased birth weight but increased the post-natal rate of growth: both results, however, were less pronounced in successive generations.

Wilkerson and Gortner (1932) from analyses of fetal pigs for their protein content have decided that the nitrogenous deposition is fixed and governed by nature and not by the maternal nutrition but it is however helped by selective placental absorption.

*Mineral metabolism in the placenta and fetus.* Recent reviews covering the analyses and mineral deposition have been made by Swanson and Iob (1939) and Gnrry and Stiven (1936) which include the recent work of Givens and Macey (1933) in the human fetus. The following general points emerge. The rate of deposition varies for different minerals, sodium being deposited fastest in the beginning and middle of pregnancy, chloride in the beginning, potassium together with calcium and phosphorus fastest toward the end of pregnancy. Ramage (1929) has devised a spectrographic method of detaching and estimating small quantities of metals. He with Sheldon and Sheldon (1933) has shown iron is stored in increasing concentration in the first six months and in the remainder of pregnancy by the total fetal weight increasing but the percentage of iron keeping constant (human), yielding an increase in the absolute weight of iron. The placental iron rises steadily but its concentration falls at full term (4.37 mgm. per cent at 7th month to 2.66 mgm. per cent at term—Wagner, 1921). Hilgenburg (1930) showed prolonged gestation still further lowers the placental iron. Mackey's

(1931) study of infant anemias conclusively proved that anemic mothers deliver themselves of babies with normal hemoglobin percentage, which however very soon develop severe hypochromic anemia, but intensive iron treatment during pregnancy prevents the onset of the anemia in the infant after birth, suggesting the fetal iron stores were low though the hemoglobin was normal. Iob and Swanson (1938) did show that the bone stores of iron were very constant but in the fetal liver there was increasing percentage. Fetzer as long ago as 1913 showed iron deficiency in the diet of the pregnant rat depleted the iron content of the fetus, often causing abortions. The assimilation is altered by accompanying food (Vahlteich, Rose and Macleod, 1936) being depressed if accompanied by calcium carbonate or other earths (Kleitzen and Kingdon, 1936). There is practically no knowledge as to the form in which iron exists in the placenta or fetus. In the chick, however, MacFarlane and Milne (1934) have found a rising percentage of the total iron from 10 per cent at the 11th day to 50 or 60 per cent at the 21st day is non-hematin iron; this presumably has come from hemoglobin katabolism since Sendju (1927) has shown that bile production is biggest after the 16th day.

Copper has been studied by MacFarlane and Milne (1934) (chick), Wilkerson (1934) (pig) and Ramage, Sheldon and Sheldon (1933) (human and goat fetuses). In the human liver the copper concentration increases steadily in percentage and, unlike iron, in the later months its percentage goes on rising concurrently with the increase in weight of the liver; at term its concentration is double that in the adult liver. In the pig the copper kept constant throughout in concentration; with the chick it rises to a maximum concentration in early life and then keeps constant. MacFarlane and Milne regard the steadily rising percentage (compared with the constancy in the pig and chick embryos) as a measure of the free diffusion of copper across the hemo-chorial placenta of the human compared with the limitation in quantity imposed by the content of the original egg and in the pig by the epitheliochorial placenta. Incidentally vitellin contains copper. There appears to be little experimental work on placental transfusion of iron or copper apart from the quantitative observational papers above. Schlossmann (1932a) regarded the mode of passage as being transfusion. Garry and Stiven (1936) have reviewed the fetal demands with reference to the maternal diet.

Manganese occurs in traces in the human fetal liver (Ramage, Sheldon and Sheldon) and it has been estimated in the calf by Gruzewska

and Roussel (1937) but no one has obtained evidence as to its form or function, beyond that it does not accumulate like iron.

Calcium is present in the human placenta and increases in absolute weight toward term but decreases in its dry weight percentage (Wehefritz, 1925). Needham discusses its presence as calcium oxalate crystals in the embryotrophe and in the hippomanes-calcified masses floating in amniotic fluid and containing glycogen of unknown significance (p. 1458). The fetal blood calcium and inorganic phosphorus vary with but are always higher than the maternal blood values. Recent analyses have been made by Mull (1936), by Bang (1937) and by Sato (1938). There is considerable evidence that the fetus makes definite demands on the maternal exogenous metabolism and if the dietary supply is defective, then upon her endogenous calcium metabolism. Dibbelt (1910) and later Zuntz in 1919 decided that in dogs and rats low calcium diets caused no deficiency of calcium in the embryo. Sontag, Munson and Hoff (1936) however found in carefully controlled experiments that diets deficient in calcium, phosphorus and vitamin D formed rat embryos short of calcium and phosphorus (gravimetrically and radiologically). On adding therapeutic doses of vitamin D the calcium and phosphorus were raised to normal levels in the litters but toxic doses of vitamin D markedly lowered the phosphorus content. It is common clinical knowledge that after pregnancy women's teeth show signs of calcium deficiency and in the absence of vitamin D osteomalacia develops in the bones. Sherman and Macleod (1925) showed in rats that pregnancy lowers the calcium and phosphorus content of the mother rat. Toverud and Toverud (1929a) showed that commonly human pregnancies are associated with negative calcium-phosphorus balances, rectifiable however by calcium administration. In a later paper they showed in dogs that this negative balance led to histological and radiological loss of calcium and if suckled while the maternal diet was still low in calcium, rickets developed accompanied by low serum calcium and phosphorus. Booker and Hansemann (1931) showed the human fetus ossifies its bones efficiently and relatively independently of the maternal intake; in fact, was parasitic on the mother. Garry and Stiven (1936) reviewing the position confirm the League of Nations report on calcium and phosphorus, and after giving clinical and experimental instances showing the demands on the maternal metabolism and diet recommend that in the last three months the calcium and phosphorus be raised from 0.68 and 1.23 grams respectively to 1 or 2 grams and 2 grams each per day. Apart from the papers of Toverud

and Toverud (1929a) and Sontag, Munson and Hoff (1936) there is little work on the effect of vitamin D in calcium-phosphorus metabolism and no evidence on the effects of parathyroid in the fetus. Burns and Henderson (1934, 1935) have shown that bone laid down in late fetal life has a low water content and a high carbon dioxide concentration and a high degree of calcification. They feel the fetal blood composition is not the most important factor controlling bone formation.

Toverud and Toverud (1929b) found magnesium injections in part rectified calcium deficiency. Further information is obtainable from Needham (1931) and Duckworth (1939).

Numerous other metals have been detected by spectroscopic methods but their rôle is not clear. (Sheldon and Ramage, 1931; Rusoff and Gaddum, 1918; Iob and Swanson, 1934.)

Shohl (1923) in his review on the maintenance of acid-base balance has pointed out the human fetus in the last two months takes daily from its mother 230 cc. of 0.1 N base and 145 cc. of 0.1 N acid radicle, a daily excess of 85 cc. of 0.1 N basic radicle.

*Vitamins and the embryo.* Maxwell reviewed the subject of vitamins and pregnancy in 1932.

*Vitamin A.* The following points seemed to be established in regard to vitamin A:

1. The placenta impedes its rapid transmission as shown by Wendt (1936) in the human and Dann (1934) in the rat.

2. It is stored in the fetal liver in high concentration especially in the early months. (Wendt, 1936; Neuweiler, 1936a and Gaehtgens, 1937a.) Gaehtgens (1937b) found a tendency to store in the placenta and Dann found increasing the fat content of the diet increased the amount which passed across the placenta. It probably accumulates wherever fat is to be found.

3. Deficiency lowers the total litter weight in rats (Korenchewsky and Carr, 1923), possibly of individual fetuses (Abels, 1922); it also produces fetal abnormalities if gross (Hale, 1933; Hughes, 1934) and fetal death which Mason (1935) shows to be due to maternal abnormalities and curable by cod liver oil. This confirms Evans (1928) who said absence of vitamin A impaired the maternal reproductive system so that fertilisation and implantation failed. This contrasts with vitamin E deficiency (see below). Green, Pindar, Davis and Mellanby (1931) showed that in pregnant women A-deficiency results in septic conditions, which can also be reproduced in rats: these effects may influence the fetus. Vitamin A concentrates increase birth weight. (Massachusetts 1938.)

*Vitamin D.* This appears to store particularly well in the human placenta (Guggisberg, 1929) and to be present in the fetal liver and in particularly high concentration in the premature infant's liver (Hess and Weinstock, 1938). Abels (1922) thought it occurred with vitamin A in excess in summer foodstuffs and influenced the birth weights where the mother was delivered in August. Abel (1931) showed it prevented rickets when given to pregnant animals and hardened fetal skull bones. Finola (1937) and Foa (1937) showed either the vitamin or sunlight increased the calcium-phosphorus deposition, thickened the skull, but did not increase phosphatase.

*Vitamin E.* Like the other fat soluble vitamins, it is held up at the placenta (Mason and Bryan, 1938). Evans, Burr and Althausen (1927) showed it was normally present in the newborn rat but that its absence allows healthy implantation of fertilised ova but there the normal pregnancy terminates. Urner (1931) demonstrated rarefaction of the fetal hematopoietic and mesenchymal tissues. Barrie (1939) has suggested its deficiency besides producing these fetal symptoms also produces maternal pregnancy toxemia (fatty infiltration of the liver, etc.). This would of course affect the nutrition of the fetus adversely.

*Vitamin B.* With rats on a minimal diet of the complex, abortion or fetal resorption is common and even if they come to birth they often die of polyneuritis (Moore and Brodie, 1927). These authors also report a case of gestational beri-beri with B 1 deficiency such as occurs in India and Portland, U. S. A. Maxwell (1932) shows latent pellagra due to lack of the pellagra-preventing factor (denoted by him in 1932 as B 2) may be unmasked by pregnancy. Neuweiler (1937) has shown that flavin has a wide distribution in the rat's fetal liver and placenta and there is no relation between age and organ content.

*Vitamin C.* A considerable amount of work has been performed since the discovery of ascorbic acid and its method of estimation. A full review of its position in pregnancy has been made by Garry and Stiven (1936). The most important points that emerge are the following.

1. Shortage in the maternal diet may cause premature births (Elmby and Becker-Christensen, 1938) and with experimental guinea pigs a deficiency in the early stages of pregnancy causes abortion and maternal scurvy but if deficient in late pregnancy the mother has no lesions of scurvy but the fetus has such lesions; however, the mother after delivery of the scorbutic young develops the symptoms of scurvy (Mouriquand, 1935, and Mouriquand, Gillet and Coeur, 1935).

2. The vitamin C content of the fetal blood is higher than that of the

mother (Wahren and Rundqvist, 1937) and both values are raised by orange juice (Manahan and Eastman, 1938) (Fleming and Sanford, 1939). Neuweiler (1935) showed there is twice as much in the blood of the umbilical vein as in that of the umbilical artery and that the fetal (human) suprarenal cortex has a higher concentration than the adult cortex. Giroud (1936a) believes synthesis of ascorbic acid occurs in animal embryos and also (1936b) in the human fetuses. Mouriquand, Coeur and Viennois (1936) feel, however, that the fetus does not synthesise vitamin C—a surprising possibility in a fetus in view of the scorbutic lesions of their earlier paper (1935b) and the absence of the power in adult animals. Neuweiler (1936b) thought that ascorbic acid is stored by the placenta and decidua and the quantity varied with the maternal diet but Muller (1939) from examinations of the cow, pig, horse and man is against storage or synthesis of ascorbic acid by the fetus.

It seems that the water soluble vitamins traverse the placenta easily in contrast to the fat soluble vitamins. On the other hand the fat soluble vitamins so far as the evidence goes seem to store in fatty organs especially the fetal liver whereas the water soluble vitamins appear to have little power of storage apart from the known peculiarity of the suprarenal cortex for concentrating ascorbic acid.

*The intermediate metabolism of the fetus.* The changes undergone by the foodstuffs have been discussed in connection with their separate metabolisms. Needham covers the work up to 1931 and since then very little has been done. Numerous enzymes have been identified but their part in the complete story is not clear and their function is not always obvious. For example, although lipase has been found in the placenta, there is no evidence that the passage of fats across it is dependent upon the presence of the enzyme. Again enzymes have been shown to develop in the fetus at different dates but the tests by which they have been identified do not show they are acting in the fetus as in the adult: as for example the presence of digestive enzymes in the fetal intestinal tract. The same generalization applies to hormones. There is a lack—owing to the difficulty of the technique—of experiments designed to show that an agent whose presence has been demonstrated by some *in vitro* or other external test, is in fact performing a physiological function in the fetus or placenta. In this connection it is of interest to note Sax and Leibson (1937) showed that after thyroidectomy fertilisation and implantation of the ovum took place followed later by its death. Development however did progress normally if it was trans-

planted to the uterus of an unthyroidectomised female. Again adrenalin has been found in most fetal suprarenals but never in that of man. Newton (1938) has reviewed the hormones of the placenta. They are confined almost entirely to the sex and related pituitary hormones. He shows there are stores of these endocrines and adduces evidence pointing to the placenta as beng a ductless gland which secretes hormones akin in activity to the known "sex" hormones.

This aspect, however, leads to the fundamental underlying development. The whole question of the nutrition of the fetus is bound up in the basic problems of the factors causing the fertilised ovum to grow, causing differentiation at different ages, causing species differences in different animals, causing development of organs before the demands of functional activity are presented. In the case of the placenta we have the interesting complication of senescent changes before the end of uterine life, a reverse of the developmental changes in earlier uterine life. The very close chemical relationships between the structure of enzymes of widely different action, such as progesterone, oesterone and androsterone, suggest that they probably are derived from an inactive precursor capable of splitting off and forming one or the other or several similar active bodies of differing action. It is not therefore surprising to find these endocrines in an autolysing organ such as the placenta. It would be of interest to know if similar products appear in autolysis elsewhere.

The metabolic errors studied by Garrod (1923) and the work of Penrose (1935, 1937) on genetic factors influencing the fetal and post-natal nutrition together with the recent work on viruses and on the related nucleoproteins indicates lines of approach to the basic problems which so far have only been touched on with regard to mammalian development.

There are certain peculiarities existent distinguishing man (and the Primata) from the other mammals in fetal and post-natal life. Rubner (1908) indicated the slow rate of growth as one. It has been shown that the high sugar content of the human milk was of the order associated with muscular activity at birth (foal, kid), which is absent in the infant, however.

In the mammal we can, taking birth weight as the principal measure of nutritional fitness, obtain the best results by breeding from good stock, by breeding from mothers of adult weight, by breeding from mothers on a high plane of nutrition. Where moderately undernourished, however, the fetal nutrition is not impaired but two things

will occur, first the maternal nutrition will be lowered still more, as shown by her condition in the puerperium (this raises the interesting problem of why it is not exhibited during pregnancy and whether the fetus exerts a controlling protecting influence on her metabolism). The second effect appears to be that although the fetal functioning metabolism is effective, the reserves are deficient, especially in regard to iron. This shortage can show itself by hypochromic anemia and post-natal malnutrition requiring extra nourishment. If the mother is grossly undernourished then does the fetus show signs before birth of malnutrition.

The fetus increases its weight mainly in the last quarter or fifth of pregnancy. It draws for minerals, for proteins and for carbohydrates on the maternal supplies, though it is not known how much comes from the mother's diet, her tissues or the autolysing placenta. It appears to build up its fat from that which it receives or to synthesise it from the carbohydrate traversing the placenta. The mother's diet if adequate does spare her tissues, but needs to be increased at this period over the non-pregnant levels of health.

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# THE CIRCULATION OF THE BILE ACIDS IN CONNECTION WITH THEIR PRODUCTION, CONJUGATION AND EXCRETION

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The entero-hepatic circulation of the bile acids has been known for a considerable time. In 1863 Hoppe-Seyler found that very little of the bile acid present in the bile could be found in the feces and he concluded that it was resorbed from the intestines. In 1870 Schiff showed the choleretic effect of bile given by mouth or directly into the intestines and he attributed this effect to the circulation of the bile. Finally Tschernoff (1884) and Stadelmann (1896) proved the resorption of bile acids from the intestines and their resorption through the liver.

Since then the circulation of the bile acids has been studied and confirmed by numerous investigators. We now know that tho bile salts are resorbed from the intestine, carried to the liver by the portal blood and again excreted with the bile. Only a small part of the bile acids is lost in each of these turns and a correspondingly small part is synthesised. If extra bile salts are supplied by intravenous injection they are almost immediately adsorbed by the surfaces of the blood-vessel system and very soon incorporated into the normal circulation. If given by mouth they also enter the circulation. Normally most of the bile acids in the bile are conjugated with glycine or taurine. If unconjugated acids are supplied they will be conjugated by the liver to a certain degree.

By the circulation of the bile acids these physiologically valuable substances are saved to a considerable extent. The same amount of bile acid may be used again and again for the resorption of fats, vitamins or other lipoids and in this way it may transport many times the amount of lipoid which it is able to keep in solution by coördination. In man the bile acids seem to pass the circulation about three times and in dogs about seven times. The significance of the bile acids for the lipoid resorption will not be discussed here.

In jaundice the normal bile circulation is blocked. If the icterus is due to damage of the liver parenchyma as in different kinds of hepatitis there is very little or no passage through the parenchyma cells to the bile capillaries. Usually the contact is better between the cells and the blood passing the liver. The inflamed liver can take up and deal with many of the substances carried to it by the portal and peripheral blood, but it has marked difficulty in absorbing several substances among which are the bile salts. For this reason these salts remain for a long time in the circulating blood if injected intravenously into hepatitic patients or animals.

In obstructive jaundice, finally, the normal bile circulation is also broken. But here the contact between the blood and the liver cells and between the liver cells and the bile capillaries is still unbroken, at least provided there is little or no secondary cell-damage. On the other hand the slight secretion pressure of the bile (in cases of low obstruction increased by the muscular pressure of the gall bladder) is enough to open the intercellular spaces between the parenchyma cells of the liver. In this way a new outflow is opened and bile can escape from the bile capillaries and reach the thoracic duct and the circulating blood via the lymph spaces and lymph vessels of the liver. Thus also in obstructive jaundice there is a kind of bile circulation. If bile salts are injected intravenously into patients or animals with obstructive jaundice the immediate disappearance is not so rapid as in normal circumstances, but the subsequent decrease in concentration is rapid as compared with that in hepatitis. This is owing to the continued capacity of the liver cells to take up the acids and to keep them circulating.

In practice (and especially in clinical cases) these two types of reaction, the hepatitic and the obstructive, are seldom quite clearly differentiated, as damage of the parenchyma usually causes an edema with some obstruction of the bile capillaries. In hepatitis, however, an opening of the lymph spaces seldom takes place unless an extensive destruction of liver cells occurs. Obstruction, on the other hand, usually causes some cell damage, sooner or later, by pressure or infection.

The literature concerning the circulation of the bile acids as also their physiology in general is rather overwhelming. Most of this literature up to 1937 is summarized in the excellent book by Sobotka.

*Analytical Methods.* Many of the studies involving analyses of the bile itself were at an early stage marked by a considerable reliability as the determinations here are easy to carry out and the methods were usually rather good. However, the numerous investigations based on

blood determinations are all of limited value, since most of the earlier methods for the determination of bile salts in the blood are very unspecific.

In the investigations by the author of the present paper and co-workers the analyses of the bile have been carried out according to previous well known methods evolved by Josephson and Jungner (1936). For the analysis of cholic acids a modification of the Gregory and Pascoe (1929) method has been used and for determinations of conjugated acids a modification of the method of Schmidt and Dart (1920/21). Concerning errors in the latter method see Doubilet and Colp (1936). The blood determinations are according to Josephson (1935) with the slight but significant modification described by Josephson and Larsson (1939). This method, like almost all of the previous ones, is nothing but a far-going modification of the old Pettenkofer (1844) test, which in its original form is not specific. However, we believe that we have proved the blood method as it is now carried out to be rather specific and with an average error of less than ± 10 per cent. It may be admitted that a small part of the color developed in the reaction may be the result of interfering non-specific substances, especially in the low normal values, but, as further emphasized by Josephson (1939b), their influence must be of very little importance. The main point of the method lies in a thorough-going isolation of the bile salts from other constituents of the blood. The determination itself depends on the well known colorimetric reaction with furfural and strong sulphuric acid which is read in the step-photometer of Pulfrich.[1] It must be clearly pointed out that by this method only cholic acid and its derivatives can be determined. No reliable method for the estimation of litho- or deoxycholic acids in blood has yet been published. Several modifications of the method mentioned above have been published recently (Abe, 1937; Lichtmann, 1938).

*The Entero Hepatic Circulation. Resorption of the bile acids from the intestine.* When the fatty acids and lipoids, combined with bile acids to soluble choleic acids (Tschernoff, 1884; Wieland and Sorge, 1916; Verzár and Kuthy, 1929) are resorbed from the intestines, the combination is again dissociated already in the mucosa cells. The fats are carried further, mainly by the lymph, but the bile acids have a tendency to stick to the intestinal walls, where they combine with new

[1] It is of special importance if one is to get good values with the method that the ethyl acetate used for the separation be pure and free from acetic acid. Unfortunately, the method is too time-consuming for routine clinical use.

fats (Verzár and Kuthy, 1929). Verzár and Kuthy (1930) found that if a bile salt solution was locked up in a piece of the small intestine a considerable part of the salts could soon be found in the wall itself, while the concentration in the fluid content decreased. The bile salts in the wall were thus assumed to act as fat transporters and in this way a small amount of salts may transport a considerable amount of fat through the mucosa even without taking part in the circulation (Riegel, O'Shea-Elsom and Ravdin, 1935).

To some extent, the bile salts themselves are also gradually carried further. When being resorbed, however, they do not follow the fats into the lymph but are transported directly to the liver by the portal blood. Thus, Josephson and Rydin (1936) always found a higher cholate concentration in the portal blood than in blood obtained by heart puncture. In rabbits and cats the concentration in blood from the portal vein was 2 to 5 mgm. per 100 ml. and in heart blood only 1 to 2 mgm. per 100 ml. As previously found by Greene, Aldrich and Rowntree (1928) this difference increases if a bile salt solution is injected into the intestine of the animal. On the other hand, Josephson and Rydin (1936) did not find any difference at all in animals having no bile in the intestine owing to a previous ligation of the common bile duct. These results were confirmed on dogs by Josephson and Kaunitz (1937) in an investigation where also the thoracic lymph was examined. In normal dogs this lymph never contained cholates in concentrations high enough to be determined even during extensive resorption of bile salts. The cholates in the portal blood of their dogs were markedly increased during bile resorption. The results have been confirmed by Jenke and Graff (1939) who found corresponding differences between blood of different origin, only with lower values throughout.

According to Tappeiner (1878), the resorption takes place chiefly in the ileum, no bile salts being resorbed in the duodenum, while only glycocholic acid in the jejunum. However, his results cannot be considered decisive, as in his experiments the intestinal mucosa was strongly irritated and bleeding (probably due to too strong alkalescense of the bile salt solutions) and the conditions were thus very unphysiological. However, his results have been confirmed by Frölicher (1935/36).

Sobotka (1937, p. 38) has made an attempt to calculate roughly the difference that might be expected between the bile salt concentration in portal and in peripherical blood, basing his results upon the bile salt excretion of bile fistula dogs according to Foster, Hooper and Whipple (1919) and found that this difference would be about 0.7 mgm. per 100

ml. of blood. He obtained the figure of the volume of blood passing the liver by calculating it to be proportional to the weight of this organ as compared with the body-weight. He also calculated the amount of bile acids produced per 24 hours and kilogram body-weight to be 0.115 gram, which is the endogenous production found by Whipple and Smith (1930) when the fistula bile was not given back by mouth to the dogs. However, Schwiegk (1932) has shown that the portal blood flow is increased by 100 to 200 per cent during resorption, and according to Whipple and Smith the bile acid excretion of fistula dogs is about 0.800 gram per kilogram body-weight when the fistula bile is re-fed to the dog which was assumed to be about the same as in normal circumstances. If these figures are used in Sobotka's formula, the difference between the bile acid content of portal blood and that of peripheral blood would be approximately about 2 mgm. per 100 ml. As the figures of Whipple and Smith (1930), confirmed by Schmidt, Beazell, Berman, Ivy and Atkinson (1939), represent the total bile acids, and as the bile acids of the dog can be considered as consisting of about two-thirds cholic acids, the difference of about 1.5 mgm. per 100 ml. found by Josephson and Kaunitz (1937) for these acids in dogs checks rather well.

*Reëxcretion of the bile acids.* Besides the studies of Tschernoff and Stadelmann, mentioned above, the entero-hepatic circulation of the bile acids has been studied by Greene, Aldrich and Rowntree (1928) and by Whipple and Smith (1928 and 1930). The latter authors found that bile fistula dogs, when deprived of their bile, produced about 100 mgm. bile salts per kilogram body weight per day. The bile salt excretion of these dogs rose to about 800 mgm. per kilogram when the dogs got all their bile back by mouth. The bile salts were then resorbed and again excreted with the fistula bile. They calculated that a dog of about 10 kgm. keeps 7 to 8 grams of bile salts in circulation by resorption and reëxcretion, and that the circulation period of this amount is about 8 to 16 hours. These results have recently been confirmed by Schmidt, Beazell, Berman, Ivy and Atkinson (1939). The circulating amount could be augmented to a certain degree by giving back to the animals more bile salts than they put out. In this way Whipple and Smith (1928) reached a bile salt secretion of about 15 to 17 grams daily, but not more. If still more bile salts were given the surplus was lost. Ivy and co-workers also found a somewhat fluctuating loss of about 10 per cent of bile salts given by mouth in physiological amounts to fistula dogs. They calculated the recovery as the surplus on top of the amount found in the fistula bile, when no bile was given by mouth. This latter

amount was supposed to represent the endogenous bile salt production. However, these authors do not reckon with the possibility that the endogenous bile salt production may be lower when bile acids are amply supplied than when the animal is continuously deprived of all newly made and all secreted bile. For this reason it is possible that the real loss is less than what they found it to be.

In this connection it may be mentioned that Doubilet (1937) found that administration by mouth of cholic acid to bile fistula dogs may depress the deoxycholic output and conversaly deoxycholic acid may decrease the cholic acid excretion by the liver, thus giving the impression of a loss of cholates.

*Loss of bile acids and endogenous production.* We do not know much about the fate of the small amounts of bile salts lost during the normal circulation. Numerous investigators (see Sobotka, 1937) claim to have found bile acids even in normal urine, but they have all used very unspecific methods. It is probable however that these salts may be excreted by the kidneys to a small extent, at least in jaundice (vide infra) or after injection of large amounts (Lichtman, 1936). Some may be destroyed by the liver (Rosenthal, Wislicki and Pommernelle, 1927; Bollman and Mann, 1933), and some may be decomposed by the intestinal bacterial flora (Licht, 1924). According to the very thorough investigations by Berman, Snapp, Ivy, Atkinson and Hough (1940) there is a possibility that some cholic acids may be oxidised by the organism to dehydro-bile acids (keto acids). In bile fistula dogs these authors found a slight increase of the keto acids in the bile if cholic acids were supplied by mouth. The main loss, however, seems to be the bile salt content of the feces shown in 1862 by Hoppe and in 1863 by Hoppe-Seyler by the means of preparation of cholic acids from dog and cow feces. We fully agree with Sobotka, when he says (p. 37): "Since catabolic destruction of bile acids by the animal organism is exceedingly doubtful, and since their elimination from the body is almost entirely confined to the intestinal route, equilibrium is maintained by synthesis of bile acids paralleling the rate of the fecal losses."

Whipple and Smith (1928) found the endogenous bile salt production of fistula dogs to be about 100 mgm. per 24 hours and kilogram bodyweight. Earlier Foster, Hooper and Whipple (1919) pointed out that bile acid production is highly dependent on the nature and quantity of the food, carbohydrates giving a low production and meat an increase. Especially tryptophan and proline were effective in bile salt production. Cholesterol, which according to its formula might be suspected as the

origin of the bile salts, was completely negative (Foster, Hooper, Whipple, 1919e; Enderlen, Thannhauser, Jenke, 1928). The chemical (structural) relationship between the bile acids and the sex hormones being well known, the biological relationship has been studied by Cuatrecasas and Bruno (1939). In hunger the bile acid production is low but still maintained (Baltaceanu and Vasiliu, 1937). Summing up the investigations in this field, we know, in fact, less than nothing about the origin of the cholic acid part of the bile acids and about the way in which the complicated molecule is being built up.

The endogenous bile salt production in man has been studied in surgical cases with bile fistulas. Greene, Walters and Fredrickson (1930/31) found the daily cholic acid output in such subjects to be 1.0 to 2.3 grams. Collecting the bile by duodenal tubage from 6 cholecystectomized but in other respects normal human subjects, Josephson and Larsson (not published) found the endogenous 24-hour production of cholic acids to be 0.7 to 2.0 grams (average 1.4). Using the same technique but with continuous refeeding of the recovered bile (vide infra) these authors found the daily excretion of 7 subjects to be 1.5 to 7.0 grams. The average was 4.0 or 2.8 times the endogenous production. This means that in man the bile acids take part in the circulation about three times.

*Injected bile salts entering the circulation.* If bile acids are supplied either by mouth or intravenously to healthy men or animals they very soon enter the normal enterohepatic circulation. At the same time they disappear from the blood almost immediately. Snell, Greene and Rowntree (1927), Bollman and Mann (1936), Chabrol, Cottet and Sallet (1936), Lichtman (1936a), Schmidt (1937) and others, all found a cholate concentration that had returned to normal within less than one hour after a large intravenous bile salt injection. Josephson, Jungner and Rydin (1938) found that most of the injected amount had disappeared already four minutes after the injection. This means that it is too late to catch the injected surplus four minutes after the injection. Normal cats and rabbits after an intravenous injection of, e.g., 250 mgm. cholic acid showed a four minute increase in the blood of only about 6 to 20 mgm. per 100 ml. If the injected amount had been diluted only by the blood of the animal the increase would have been about 100 mgm. per 100 ml. When only 10 mgm. per kilogram body-weight were injected the corresponding increase was 1 to 3 mgm. instead of the calculated 10. Josephson and Larsson (1939) and Josephson (1939a) got similar results on man (fig. 4). Five minutes after the injection of 1000 mgm. cholic

acid the blood content had risen from the normal 2 to 3 mgm. per 100 ml. to 5 to 6 mgm. per 100 ml. and after 500 mgm. it rose to about 4 mgm. per 100 ml. The corresponding dilution values were very approximately about 22 and 12 mgm. per 100 ml. As a rule the blood concentration was normal again after 30 minutes.

In normal individuals the injected salts are soon excreted by the liver. But their first rapid disappearance from the blood cannot be referred solely to this organ. Even if the liver was excluded by ligation of the liver artery and the portal vein, the concentration four minutes after an injection was still only about two-thirds of what might have been expected if the bile salts had been only diluted by the blood (Josephson, Jungner and Rydin, 1938). The most likely explanation of this disappearance is diffusion of the salts into the tissues and fixation there. Bayer (1908) and Chabrol, Cottet and Sallet (1936b) found a fixation of this kind in liver and muscle tissue. On the other hand, Jungner, Rydin and Josephson (1938), in experiments on cats with both obstructive jaundice and liver exclusion, found no disappearance of injected cholates at all. The enormous blood concentrations in these cases, 100 to 400 mgm. per 100 ml. after injection of 250 mgm., roughly corresponded to the dilution of the injected solution by the blood of the animal. Comparison of the results on liver-excluded animals with and without jaundice seems to indicate a fixation mechanism in the normal individual which is not present in jaundice. Possibly this mechanism may be a fixation of the bile salts, which are strongly surface active, to the walls of the blood vessels. This fixation may be of the same type as that found by Verzár and Kuthy (1930), mentioned above, on the walls of the intestine or by Labes and Schlenkert (1932) on red cells and other tissues.

The rapid disappearance of injected bile salts from the blood is normally followed by an almost quantitative excretion by the liver. This has been proved by numerous investigators (see Sobotka and more recently Bollman and Mann, 1936, and Josephson, Jungner and Rydin, 1938). In patients or animals with bile fistulas, 90 to 100 per cent of a not too large amount of injected bile salts is recovered in the bile a few hours after the injection (fig. 1).

The results obtained with bile fistulas may not be considered as true physiological observations. However, Josephson and Larsson (1939) showed that the same rule holds good also when the bile is emptied into the intestine in the normal way. They used six human subjects, who were normal in all respects, except that they had been cholecystectomised one or several years before the experiment. It was necessary to choose

such subjects in order to exclude the function of the gall bladder and to get a continuous bile flow. The bile was quantitatively recovered by means of a double ventriculo-duodenal tube. One gram of sodium

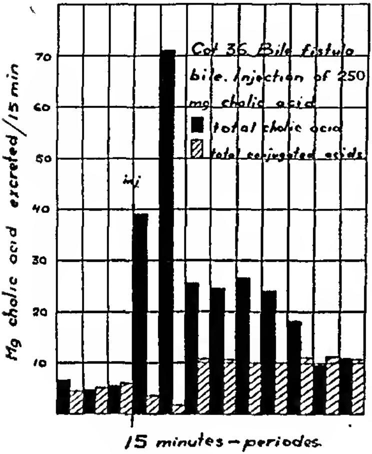


Fig. 1

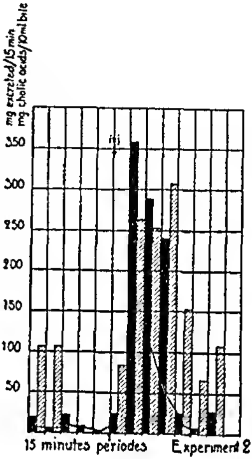


Fig. 2

Fig. 1. From figures of Josephson, Jungner, Rydin (1938). Recovery of cholic acids (black columns) and conjugated bile acids (striped columns) in the bile after intravenous injection of 250 mgm. cholic acid. Two hundred and ten milligram cholic acids recovered in 3 hours. Increase of cholic acid excretion starts immediately after the injection; increase of conjugated acids starts half an hour later.

Fig. 2. From Josephson and Larsson (1939). Recovery by duodenal tubage of cholic acids (black columns) and conjugated bile acids (striped columns) in the bile of a 59 years old, cholecystectomized woman after intravenous injection of 1 gram of cholic acid. The black line is concentration in the bile; 0.88 gram cholic acid recovered in 4 hours. Increase of both cholic and conjugated acids 15 minutes after the injection, but conjugation is incomplete during at least half an hour.

cholate was injected intravenously and after four hours 0.9 to 1.1 gram was recovered in the bile in four cases and 0.5 in two cases (fig. 2).

It seems obvious that the normal liver works very promptly on bile

investigation Josephson, Jungner and Rydin (1938), using comparatively larger amounts of cholic acid, obtained different results. They injected 250 mgm. cholic acid in the form of sodium cholate intravenously into cats and rabbits provided with a cannula in the common bile duct and with the gall bladder ligated. During the first 30 minutes after the injection a comparatively enormous bile salt excretion took place, but nearly all the excreted cholate was unconjugated (fig. 1). Later on, when the bile salt excretion was slowly decreasing, more and more of the cholate appeared in the conjugated form. Corresponding results were obtained by Josephson and Larsson (1939) on human subjects (fig. 2).

*The nature of the conjugation.* These results might be expected, as the conjugation of the bile salts must be an enzymatic process and thus requires time. Josephson, Jungner and Rydin (1938) even considered this observation to be evidence of the enzymatic character of the conjugation of the bile acids. In fact, Mazza and Stolfi (1932) had earlier claimed that they had prepared an enzyme with this property from liver tissue.

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*Bile acid conjugation and protein formation.* The rôle of the glycine and the taurine in these conjugation processes, the formation of hippuric acid and that of conjugated bile acids, is very interesting. Considering the question as to why just glycine can be conjugated with benzoic acid we have carried out some further experiments in this laboratory on the formation of peptid linkages. First, we were able to confirm the observation made on rabbits by Griffith and Lewis (1923) and on man by Probstein and Londe (1940), that the excretion of hippuric acid is much higher when glycine is given together with the benzoic acid, than when benzoic acid is given alone. Secondly, we were also able to confirm in man the observation made on rabbits by Griffith and Lewis (1923) that administration of other amino acids with the benzoic acid does not produce any excretion of their benzoyl compounds. So far the amino acids d-alanine and l-histidine have been tested. Thirdly, we are now giving patients with a poor hippuric acid excretion dipeptides containing glycine as one of the components at the same time as the benzoic acid is given. The benzoic acid is, as usual, given by mouth, but the di-peptides are given intravenously in order to prevent their cleavage by the digestive enzymes. It seems as if glycine were the key substance for the formation of peptide linkages and the presence of its free carboxyl group were necessary for the start of the synthesis of a peptide chain. With the carboxyl group of glycine at one end of the chain, more amino acids can always be added to the other end. When a cholic acid or benzoic acid is linked to the amino group of the glycine as in glycocholic or hippuric acid, the building up of the chain is abruptly cut off, as these substances have no amino group. On the

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blood determinations are all of limited value, since most of the earlier methods for the determination of bile salts in the blood are very unspecific.

In the investigations by the author of the present paper and co-workers the analyses of the bile have been carried out according to previous well known methods evolved by Josephson and Jungner (1936). For the analysis of cholic acids a modification of the Gregory and Pascoe (1929) method has been used and for determinations of conjugated acids a modification of the method of Schmidt and Dart (1920/21). Concerning errors in the latter method see Doubilet and Colp (1936). The blood determinations are according to Josephson (1935) with the slight but significant modification described by Josephson and Larsson (1939). This method, like almost all of the previous ones, is nothing but a far-going modification of the old Pettenkofer (1844) test, which in its original form is not specific. However, we believe that we have proved the blood method as it is now carried out to be rather specific and with an average error of less than $\pm$ 10 per cent. It may be admitted that a small part of the color developed in the reaction may be the result of interfering non-specific substances, especially in the low normal values, but, as further emphasized by Josephson (1939b), their influence must be of very little importance. The main point of the method lies in a thorough-going isolation of the bile salts from other constituents of the blood. The determination itself depends on the well known colorimetric reaction with furfural and strong sulphuric acid which is read in the step-photometer of Pulfrich.[1] It must be clearly pointed out that by this method only cholic acid and its derivatives can be determined. No reliable method for the estimation of litho- or deoxycholic acids in blood has yet been published. Several modifications of the method mentioned above have been published recently (Abe, 1937; Lichtmann, 1938).

*The Entero Hepatic Circulation. Resorption of the bile acids from the intestine.* When the fatty acids and lipoids, combined with bile acids to soluble choleic acids (Tschernoff, 1884; Wieland and Sorge, 1916; Verzár and Kuthy, 1929) are resorbed from the intestines, the combination is again dissociated already in the mucosa cells. The fats are carried further, mainly by the lymph, but the bile acids have a tendency to stick to the intestinal walls, where they combine with new

[1] It is of special importance if one is to get good values with the method that the ethyl acetate used for the separation be pure and free from acetic acid. Unfortunately, the method is too time-consuming for routine clinical use.

fats (Verzár and Kuthy, 1929). Verzár and Kuthy (1930) found that if a bile salt solution was locked up in a piece of the small intestine a considerable part of the salts could soon be found in the wall itself, while the concentration in the fluid content decreased. The bile salts in the wall were thus assumed to act as fat transporters and in this way a small amount of salts may transport a considerable amount of fat through the mucosa even without taking part in the circulation (Riegel, O'Shea-Elsom and Ravdin, 1935).

To some extent, the bile salts themselves are also gradually carried further. When being resorbed, however, they do not follow the fats into the lymph but are transported directly to the liver by the portal blood. Thus, Josephson and Rydin (1936) always found a higher cholate concentration in the portal blood than in blood obtained by heart puncture. In rabbits and cats the concentration in blood from the portal vein was 2 to 5 mgm. per 100 ml. and in heart blood only 1 to 2 mgm. per 100 ml. As previously found by Greene, Aldrich and Rowntree (1928) this difference increases if a bile salt solution is injected into the intestine of the animal. On the other hand, Josephson and Rydin (1936) did not find any difference at all in animals having no bile in the intestine owing to a previous ligation of the common bile duct. These results were confirmed on dogs by Josephson and Kaunitz (1937) in an investigation where also the thoracic lymph was examined. In normal dogs this lymph never contained cholates in concentrations high enough to be determined even during extensive resorption of bile salts. The cholates in the portal blood of their dogs were markedly increased during bile resorption. The results have been confirmed by Jenke and Graff (1939) who found corresponding differences between blood of different origin, only with lower values throughout.

According to Tappeiner (1878), the resorption takes place chiefly in the ileum, no bile salts being resorbed in the duodenum, while only glycocholic acid in the jejunum. However, his results cannot be considered decisive, as in his experiments the intestinal mucosa was strongly irritated and bleeding (probably due to too strong alkalescense of the bile salt solutions) and the conditions were thus very unphysiological. However, his results have been confirmed by Frölicher (1935/36).

Sobotka (1937, p. 38) has made an attempt to calculate roughly the difference that might be expected between the bile salt concentration in portal and in peripherical blood, basing his results upon the bile salt excretion of bile fistula dogs according to Foster, Hooper and Whipple (1919) and found that this difference would be about 0.7 mgm. per 100

ml. of blood. He obtained the figure of the volume of blood passing the liver by calculating it to be proportional to the weight of this organ as compared with the body-weight. He also calculated the amount of bile acids produced per 24 hours and kilogram body-weight to be 0.115 gram, which is the endogenous production found by Whipple and Smith (1930) when the fistula bile was not given back by mouth to the dogs. However, Schwiegk (1932) has shown that the portal blood flow is increased by 100 to 200 per cent during resorption, and according to Whipple and Smith the bile acid excretion of fistula dogs is about 0.800 gram per kilogram body-weight when the fistula bile is re-fed to the dog which was assumed to be about the same as in normal circumstances. If these figures are used in Sobotka's formula, the difference between the bile acid content of portal blood and that of peripheral blood would be approximately about 2 mgm. per 100 ml. As the figures of Whipple and Smith (1930), confirmed by Schmidt, Beazell, Berman, Ivy and Atkinson (1939), represent the total bile acids, and as tho bile acids of the dog can be considered as consisting of about two-thirds cholic acids, the difference of about 1.5 mgm. per 100 ml. found by Josephson and Kaunitz (1937) for these acids in dogs checks rather well.

*Reëxcretion of the bile acids.* Besides the studies of Tschernoff and Stadelmann, mentioned above, the entero-hepatic circulation of the bile acids has been studied by Greene, Aldrich and Rowntree (1928) and by Whipple and Smith (1928 and 1930). The latter authors found that bile fistula dogs, when deprived of their bile, produced about 100 mgm. bile salts per kilogram body weight per day. The bile salt excretion of these dogs rose to about 800 mgm. per kilogram when the dogs got all their bile back by mouth. The bile salts were then resorbed and again excreted with the fistula bile. They calculated that a dog of about 10 kgm. keeps 7 to 8 grams of bile salts in circulation by resorption and reëxcretion, and that the circulation period of this amount is about 8 to 16 hours. These results have recently been confirmed by Schmidt, Beazell, Berman, Ivy and Atkinson (1939). The circulating amount could be augmented to a certain degree by giving back to the animals more bile salts than they put out. In this way Whipple and Smith (1928) reached a bile salt secretion of about 15 to 17 grams daily, but not more. If still more bile salts were given the surplus was lost. Ivy and co-workers also found a somewhat fluctuating loss of about 10 per cent of bile salts given by mouth in physiological amounts to fistula dogs. They calculated the recovery as the surplus on top of the amount found in the fistula bile, when no bile was given by mouth. This latter

amount was supposed to represent the endogenous bile salt production. However, these authors do not reckon with the possibility that the endogenous bile salt production may be lower when bile acids are amply supplied than when the animal is continuously deprived of all newly made and all secreted bile. For this reason it is possible that the real loss is less than what they found it to be.

In this connection it may be mentioned that Doubilet (1937) found that administration by mouth of cholic acid to bile fistula dogs may depress the deoxycholic output and conversaly deoxycholic acid may decrease the cholic acid excretion by the liver, thus giving the impression of a loss of cholates.

*Loss of bile acids and endogenous production.* We do not know much about the fate of the small amounts of bile salts lost during the normal circulation. Numerous investigators (see Sobotka, 1937) claim to have found bile acids even in normal urine, but they have all used very unspecific methods. It is probable however that these salts may be excreted by the kidneys to a small extent, at least in jaundice (vide infra) or after injection of large amounts (Lichtman, 1936). Some may be destroyed by the liver (Rosenthal, Wislicki and Pommernelle, 1927; Bollman and Mann, 1933), and some may be decomposed by the intestinal bacterial flora (Licht, 1924). According to the very thorough investigations by Berman, Snapp, Ivy, Atkinson and Hough (1940) there is a possibility that some cholic acids may be oxidised by the organism to dehydro-bile acids (keto acids). In bile fistula dogs these authors found a slight increase of the keto acids in the bile if cholic acids were supplied by mouth. The main loss, however, seems to be the bile salt content of the feces shown in 1862 by Hoppe and in 1863 by Hoppe-Seyler by the means of preparation of cholic acids from dog and cow feces. We fully agree with Sobotka, when he says (p. 37): "Since catabolic destruction of bile acids by the animal organism is exceedingly doubtful, and since their elimination from the body is almost entirely confined to the intestinal route, equilibrium is maintained by synthesis of bile acids paralleling the rate of the fecal losses."

Whipple and Smith (1928) found the endogenous bile salt production of fistula dogs to be about 100 mgm. per 24 hours and kilogram body-weight. Earlier Foster, Hooper and Whipple (1919) pointed out that bile acid production is highly dependent on the nature and quantity of the food, carbohydrates giving a low production and meat an increase. Especially tryptophan and proline were effective in bile salt production. Cholesterol, which according to its formula might be suspected as the

origin of the bile salts, was completely negative (Foster, Hooper, Whipple, 1919c; Enderlen, Thannhauser, Jenke, 1928). The chemical (structural) relationship between the bile acids and the sex hormones being well known, the biological relationship has been studied by Cuatrecasas and Bruno (1939). In hunger the bile acid production is low but still maintained (Baltaceanu and Vasiliu, 1937). Summing up the investigations in this field, we know, in fact, less than nothing about the origin of the cholic acid part of the bile acids and about the way in which the complicated molecule is being built up.

The endogenous bile salt production in man has been studied in surgical cases with bile fistulas. Greene, Walters and Fredrickson (1930/31) found the daily cholic acid output in such subjects to be 1.0 to 2.3 grams. Collecting the bile by duodenal tubage from 6 cholecystectomized but in other respects normal human subjects, Josephson and Larsson (not published) found the endogenous 24-hour production of cholic acids to be 0.7 to 2.0 grams (average 1.4). Using the same technique but with continuous refeeding of the recovered bile (vide infra) these authors found the daily excretion of 7 subjects to be 1.5 to 7.0 grams. The average was 4.0 or 2.8 times the endogenous production. This means that in man the bile acids take part in the circulation about three times.

*Injected bile salts entering the circulation.* If bile acids are supplied either by mouth or intravenously to healthy men or animals they very soon enter the normal enterohepatic circulation. At the same time they disappear from the blood almost immediately. Snell, Greene and Rowntree (1927), Bollman and Mann (1936), Chabrol, Cottet and Sallet (1936), Lichtman (1936a), Schmidt (1937) and others, all found a cholate concentration that had returned to normal within less than one hour after a large intravenous bile salt injection. Josephson, Jungner and Rydin (1938) found that most of the injected amount had disappeared already four minutes after the injection. This means that it is too late to catch the injected surplus four minutes after the injection. Normal cats and rabbits after an intravenous injection of, e.g., 250 mgm. cholic acid showed a four minute increase in the blood of only about 6 to 20 mgm. per 100 ml. If the injected amount had been diluted only by the blood of the animal the increase would have been about 100 mgm. per 100 ml. When only 10 mgm. per kilogram body-weight were injected the corresponding increase was 1 to 3 mgm. instead of the calculated 10. Josephson and Larsson (1939) and Josephson (1939a) got similar results on man (fig. 4). Five minutes after the injection of 1000 mgm. cholic

acid the blood content had risen from the normal 2 to 3 mgm. per 100 ml. to 5 to 6 mgm. per 100 ml. and after 500 mgm. it rose to about 4 mgm. per 100 ml. The corresponding dilution values were very approximately about 22 and 12 mgm. per 100 ml. As a rule the blood concentration was normal again after 30 minutes.

In normal individuals the injected salts are soon excreted by the liver. But their first rapid disappearance from the blood cannot be referred solely to this organ. Even if the liver was excluded by ligation of the liver artery and the portal vein, the concentration four minutes after an injection was still only about two-thirds of what might have been expected if the bile salts had been only diluted by the blood (Josephson, Jungner and Rydin, 1938). The most likely explanation of this disappearance is diffusion of the salts into the tissues and fixation there. Bayer (1908) and Chabrol, Cottet and Sallet (1936b) found a fixation of this kind in liver and muscle tissue. On the other hand, Jungner, Rydin and Josephson (1938), in experiments on cats with both obstructive jaundice and liver exclusion, found no disappearance of injected cholates at all. The enormous blood concentrations in these cases, 100 to 400 mgm. per 100 ml. after injection of 250 mgm., roughly corresponded to the dilution of the injected solution by the blood of the animal. Comparison of the results on liver-excluded animals with and without jaundice seems to indicate a fixation mechanism in the normal individual which is not present in jaundice. Possibly this mechanism may be a fixation of the bile salts, which are strongly surface active, to the walls of the blood vessels. This fixation may be of the same type as that found by Verzár and Kuthy (1930), mentioned above, on the walls of the intestine or by Labes and Schlenkert (1932) on red cells and other tissues.

The rapid disappearance of injected bile salts from the blood is normally followed by an almost quantitative excretion by the liver. This has been proved by numerous investigators (see Sobotka and more recently Bollman and Mann, 1936, and Josephson, Jungner and Rydin, 1938). In patients or animals with bile fistulas, 90 to 100 per cent of a not too large amount of injected bile salts is recovered in the bile a few hours after the injection (fig. 1).

The results obtained with bile fistulas may not be considered as true physiological observations. However, Josephson and Larsson (1939) showed that the same rule holds good also when the bile is emptied into the intestine in the normal way. They used six human subjects, who were normal in all respects, except that they had been cholecystectomised one or several years before the experiment. It was necessary to choose

such subjects in order to exclude the function of the gall bladder and to get a continuous bile flow. The bile was quantitatively recovered by means of a double ventriculo-duodenal tube. One gram of sodium

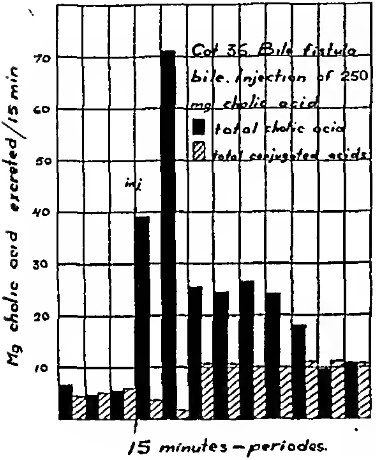


Fig. 1

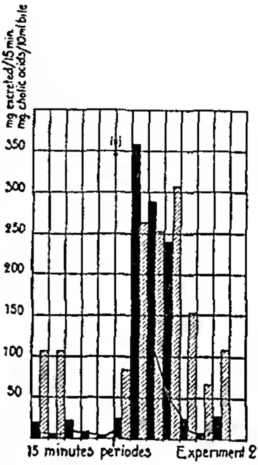


Fig. 2

Fig. 1. From figures of Josephson, Jungner, Rydin (1938). Recovery of cholic acids (black columns) and conjugated bile acids (striped columns) in the bile after intravenous injection of 250 mgm. cholic acid. Two hundred and ten milligram cholic acids recovered in 3 hours. Increase of cholic acid excretion starts immediately after the injection; increase of conjugated acids starts half an hour later.

Fig. 2. From Josephson and Larsson (1939). Recovery by duodenal tubage of cholic acids (black columns) and conjugated bile acids (striped columns) in the bile of a 59 years old, cholecystectomized woman after intravenous injection of 1 gram of cholic acid. The black line is concentration in the bile; 0.88 gram cholic acid recovered in 4 hours. Increase of both cholic and conjugated acids 15 minutes after the injection, but conjugation is incomplete during at least half an hour.

cholate was injected intravenously and after four hours 0.9 to 1.1 gram was recovered in the bile in four cases and 0.5 in two cases (fig. 2).

It seems obvious that the normal liver works very promptly on bile

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other hand, the free amino-group of glycine does not seem to be sufficient for the start of the synthesis if the carboxyl group is conjugated. The experiments are being continued and will be published later.

*The Continuity of the Bile Acid Excretion. Diurnal variations in the liver function.* Very little is known about the continuity of the excretion and formation of the bile acids. Forsgren (1928) discovered the rhythm of the liver function. His discovery was followed by a series of investigations in this field, mostly in Swedish laboratories. Using histological methods Forsgren (1928) found that the glycogen content of the liver of rabbits was highest during the night and in the early morning, the assimilatory phase. The content of what he called bile constituents was highest during the day and evening, the secretory phase. There were in different animals slight variations in the hours when the phases appeared (Forsgren, 1930). The bile constituents were demonstrated only by the formation of microscopically visible precipitation with barium chloride, and for this reason it cannot be considered as proved that they included the bile acids. However, the results have been confirmed later by different methods. The rhythm of the glycogen content has been demonstrated by Ågren, Wilander and Jorpes (1931) and by Sjoegren, Nordenskjoeld, Holmgren and Moellerstroem (1938). The latter investigators found the variations to be largely dependent on food intake. Using chemical methods for the study of the variations of the blood sugar concentration in man, Moellerstroem (1930) concluded that a similar rhythm occurs also in the human liver. Sechel and Kato (1938) using newborn rats and the Forsgren methods found no liver rhythm during the first three weeks of life. After that age the rhythm was developed. Holmgren (1936) in an extensive histological study of the rhythmic changes in the resorption from the intestines, found variations of the fat content in the intestinal mucosa speaking in favour of Forsgren's opinion that there are rhythmic changes also in the bilt salt secretion. These changes are probably followed by changes in the resorption of fat acids, and in this way they have an influence on the histological picture of the mucosa. Higgins, Berkson and Flock (1933) emphasized that the cyclic changes in the rat liver are entirely due to the time of food intake. They were able to change the time of the maximum and minimum of the liver glycogen by changing the time of the meals. But even from their experiments some endogenous cyclic changes can be inferred.

There are some direct observations published on the daily variations in bile flow and bile acid excretion in patients and animals with bile

fistulas. In 1889 Copeman and Winston and in 1897 Pfaff and Balch found that patients with bile fistulas produced more bile during the daytime and evening than during the night and morning. These observations are in conformity with the later observations by Forsgren (1928). They did not include bile acid analysis. Foster, Hooper and Whipple (1919a) mentioned that the bile salt output of bile fistula dogs is somewhat higher in the morning than in the evening.

Josephson and Larsson (1934) studied a bile fistula patient. Concerning the variations in the bile volume they confirmed the earlier result, but they found another rhythm in the bile acid output. In this patient a weak excretion maximum was observed about midnight. The variations in bile volume output in man have later been confirmed by Koster, Shapiro and Lerner (1936) and by Zuckermann, Kogut and Jacobi (1939).

*Diurnal variations of the bile acid secretion.* All studies of the variations in the bile salt excretion of bile fistula subjects have the drawback that they do not correspond to physiological conditions when the bile acids are circulating. In the hope of approaching what normally occurs, Josephson and Larsson (not published) undertook some experiments in this field. They used human subjects, on whom a cholecystectomy had been carried out some years earlier. They chose cases in which the operation had been without complications, and which were quite normal when the experiments were carried out. The use of cholecystectomized patients was necessary in order to obtain the liver bile constantly without interruptions from the gall bladder. The bile was continuously collected by weak suction through a double duodenal tube with one of the branches in the stomach and one in the duodenum. The subjects fasted throughout the experiments, which lasted during 24 to 30 hours.

In the first series of experiments the bile was continuously withdrawn. In another series on the same subjects the bile was collected during 30 minutes each hour, a sample was taken for analysis and the bile was slowly reinjected through the duodenal tube during the other 30 minutes of the same hour. In this way a physiological entero-hepatic circulation could be maintained during the whole experiment. At the same time the bile was continuously analysed and quantitatively measured.

In both series the values varied extremely from hour to hour and no typical or constantly returning maxima or minima could be found. However, the excretion of bile and cholates tended to be greater during

the day, especially the forenoon, than during the night. This difference was observed in both series but was more striking in the experiments where the bile was reinjected. Consequently, there is apparently a rhythmic variation, at least of the excretion or entero-hepatic circulation of the cholates in man, principally of the type emphasized by Forsgren (1928). If this rhythm is to be credited variations in the liver function or in the resorption from the intestine cannot be definitely decided, but the latter reason seems to be the most probable one, as the bile acids are very soon reëxcreted when once introduced into the blood, as described above. The existence of rhythmic variations in the endogenous bile acid production is very doubtful.

The results arrived at by Josephson and Larsson will be published later in full detail, but a picture of their results concerning the excretion of the bile acids is demonstrated in figure 3, showing two typical experiments.

*Liver Diseases and Bile Acid Circulation. Bile salt production in jaundice.* In jaundice, whether obstructive or caused by parenchymatous damage of the liver cells (hepatitis), the entero-heptic circulation of the bile acids is more or less completely broken.

In obstruction the liver cells still maintain their ability to produce and to transport bile acids. Their concentration in the blood is increased and they also appear in the urine. If the liver parenchyma is injured the bile acid production decreases. Thus the bile acid excretion in the urine of dogs with the bile ducts ligated is diminished if the liver cells of the animals are damaged by poisons, for instance, by carbontetrachloride (Bollman and Mann, 1935) and the bile salt excretion by bile fistula dogs is also diminished by such poisons (Whipple and Smith, 1930). A similar reaction is observed in patients with disorders of the bile ducts or the gall bladder. After operation including the establishment of a bile fistula these patients usually have a very low bile salt production during the first three or four days following the operation (Colp and Doubilet, 1936; Kohlstaedt and Helmer, 1937; Grey, Butsch and McGowan, 1938). When the liver has recovered the production is normal again.

In jaundice there is usually an increased concentration of the bile acids in the blood. The figures published by the numerous investigators on this subject (Snell, Green, Rowntree, 1927; Katayama, 1928; Fuentes, Apolo and Esculies, 1930; Bollman and Mann, 1935; Jungner, Rydin and Josephson, 1938, and others) vary considerably, according to the different methods of analysis. Usually the figures are higher in ob-

structive jaundice (up to 30 mgm. per 100 ml.) than in hepatitis (up to 10 mgm. per 100 ml.) partly owing to the maintained ability of the

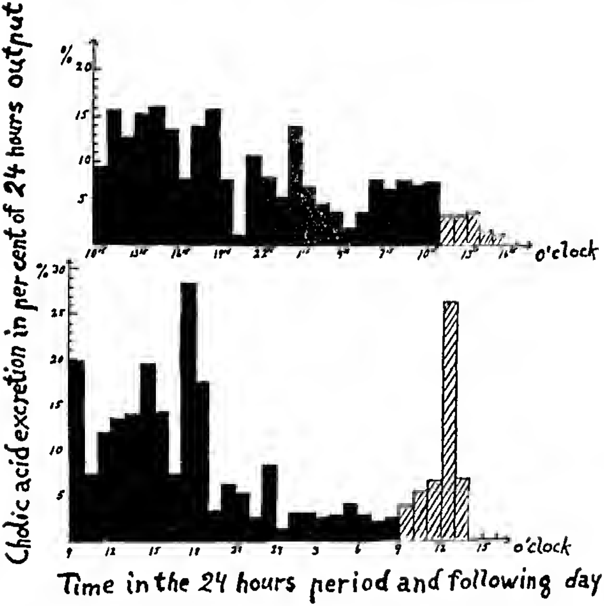


Fig. 3. From Josepbson and Larsson (not published). Diurnal excretion of cholic acids in two healthy cholecystectomized women, one 26 years old (above) and ono 18 years old (below). The bile was collected by duodenal tube during tbe first 30 minutes of each hour. During the other 30 minutes, after a sample had first been taken, tbe bile was slowly re-injected into the duodenum, thus maintaining tbs normal circulation. Both experiments lasted for 29 hours, the first 24 demonstrated by black columns, the last 5 by striped columns. During this period tbe patients were in bed and got neither food nor drink. Considerably more cholic acids excreted during the day than during the night.

liver cells in obstruction to produce bile salts and partly to the hepato-bilio-lymphatic circulation appearing only in obstruction which carries the bile salts from the liver to the circulating blood.

*Bile salt circulation in jaundice.* In cases of obstruction a new circulation route takes the place of the entero-hepatic one. Mendel and Underhill (1905), and even investigators previous to them, proved that in animals with the common bile duct ligated, substances present in the bile (injected dyes or milk) could be traced along the lymph vessels of the liver and in the thoracic duct, where these vessels open out. Mendel and Underhill consequently suspected this to be the route by which the bile constituents reach the blood in jaundice. This is in conformity with later clinical and pathological observations described for instance by Eppinger (1937) in his discussion of what he calls "icterus e dissociatione." Recently Shafiroff, Doubilet and Ruggiero (1939) have found that the bile pigments can follow the same route. Applying a slight pressure of about 30 cm. water (in the form of a bile column) on the content of the hepatic duct of dogs with the common bile duct and gall bladder ligated, they found bile pigments in the thoracic duct lymph after 10 to 20 minutes.

Josephson and Kaunitz (1937) showed that in obstructive jaundice also the cholic acids make such a hepato-bilio-lymphatic circulation. These authors used dogs in acute experiments with a fistula to the thoracic duct. They found that animals in which the normal outflow of bile was maintained had no cholates in the lymph. On the other hand, if the animals were icteric, owing to a previous ligation of the common bile duct, they found a considerable cholate concentration in the lymph. This concentration increased still more if these dogs were given taurocholates by mouth. Obviously, the bile acids had been resorbed from the intestine, carried to the liver by the portal blood and had entered the hepato-bilio-lymphatic circulation.

Doubilet and Colp (1936) spoke of another form of circulation of bile salts in jaundice. They found that in cholecystitis or cholangitis about nine-tenths of the bile salts, especially the cholates, was reabsorbed from the gall bladder and the bile ducts and again carried to the liver. Normally, this simple bilio-hepatic circulation does not occur. In cholecystitis it takes up to 90 per cent of the cholates.

In hepatitis and other forms of damage to the liver cells themselves the diseased cells form a barrier between the blood and the bile capillaries. For this reason no circulation of bile constituents whatsoever takes place. This may possibly be one of the reasons why the bile acids in the bile from fistula dogs decrease when the animals are given carbontetrachloride or other liver-poisons. Of course a more important reason may be diminished bile salt formation in hepatitis.

The difference between the circulation of the bile salts in obstruction and the blockage in hepatitis may be illustrated by the different behavior of cholates injected intravenously.

If bile salts are injected intravenously into icteric animals or patients the resulting increase of the cholates in the blood is much higher and lasts longer than is normally the case (Snell, Green and Rowntree, 1927; Chabrol, Cottet and Sallet, 1936a; Bollman and Mann, 1936; Jungner, Rydin and Josephson, 1938). However, also in this case the main part of the injected salts very soon disappears from the blood. The fate of the bile salts after their disappearance has been the subject of several theories; Rosenthal, Wislicki and Pommernelle (1927) and Bollman and Mann (1936) claim that the acids are partly destroyed by the liver. Bollman and Mann (1933) regard their observation that supplied bile acids appeared in the urine after complete removal of the liver but not when the liver was intact and the bile duct ligated, as in favour of their theory of destruction in the liver. However, their urine method is extremely unspecific. Chabrol, Cottet and Sallet (1936b) recovered most of the injected salts in the liver tissue of the jaundiced animal and concluded that the liver has a special property of fixing and storing the bile salts. Finally, as indicated by the Hay test, the surplus may be excreted by the kidneys. Thus, attempts to determine the bile salts in the urine have been made by numerous authors with positive results (Bollman and Mann, 1936; Lichtman, 1936), but they all used too unspecific methods of analysis. According to Lichtman the cholates should pass the kidneys easier than the deoxycholates, thus causing a concentration of deoxycholic acid in the blood higher than that of cholic acid.

Jungner, Rydin and Josephson (1938) claimed that injected bile salts in obstructive jaundice are absorbed by the liver according to Chabrol, Cottet and Sallet (1936b) but that little by little they subsequently enter the hepato-bilio-lymphatic circulation. This is the reason why injected bile salts disappear comparatively quickly from the blood in obstruction. In hepatic jaundice on the other hand where there is no bile circulation they remain for a long time in the blood. An adsorption to the walls of the blood vessels (as mentioned above) does not seem to take place in jaundice, at least not in the same degree as normally. This is supported by the above-mentioned observations of Josephson, Jungner and Rydin that if an injection of bile salts is undertaken on an animal with the liver excluded and having a considerable icterus due to a previous ligation of the bile duct, the blood con-

centration will rise enormously and roughly correspond to the simple dilution of the injected solution by the blood of the animal. In these cases the walls were already loaded with bile salts owing to the ligation and the subsequent jaundice.

*Diagnostic use of bile acid elimination.* From a diagnostic point of view Jungner, Rydin and Josephson (1936) studied the elimination from the blood of cholic acids injected intravenously into animals with several forms of experimental jaundice (ligation, carbontetrachlorid, phosphorus). They found that in obstruction the injected bile acids disappeared from the blood rather quickly. Sometimes a new, slight increase occurred again about one hour after the injection. Jungner, Rydin and Josephson (1936) explained this by the maintenance of the hepato-biliolymphatic circulation, by which the bile salts are again carried to the blood, thus causing the second peak. In hepatitis the decrease of the cholate concentration of the blood was rather slow after the first rise in connection with an injection.

Josephson (1939a) has tried to utilise these different types of reaction in a new liver function test on patients with icterus. He injected 10 ml. of a 0.52 per cent sodium cholate solution (corresponding to 0.5 gram cholic acid) intravenously into patients with disorders of the liver or bile ducts. The solution also contained 25 per cent glucose, which prevents the burning sensation following an injection of a pure cholate solution. The blood cholates were determined both before and 5, 30 and 60 minutes after the injection. It turned out that the disappearance of the cholate from the blood in the different types of liver disease was in conformity with the results observed in animals with experimental liver injuries. Some typical results are shown in figure 4.

In cases with liver disease without jaundice (some cases of liver cancer, cholecystitis and cirrhosis) the cholates disappeared quite as rapidly as in normal subjects. After 5 minutes only a slight elevation could be found and after 30 minutes the blood concentration was normal again. In bile obstruction the increase 5 minutes after the injection was usually obvious, 5 to 8 mgm. per 100 ml., but after 30 minutes the concentration had already fallen to the original level or almost so. In cases with damage of the liver parenchyma (mainly hepatitis) on the other hand the first increase after the injection was usually of the same order of magnitude as in obstructive jaundice. The subsequent decrease of cholates, however, was very slow in hepatitis in contrast to its behaviour in obstruction. Even one hour after the injection the concentration of the blood cholates in hepatitis was still nearly as high as after 5

minutes. From this it may be assumed that the rate of the elimination of injected cholate may be used in the differential diagnosis between obstructive jaundice and disease of the liver parenchyma such as hepatitis. A difference between the concentration 5 minutes after

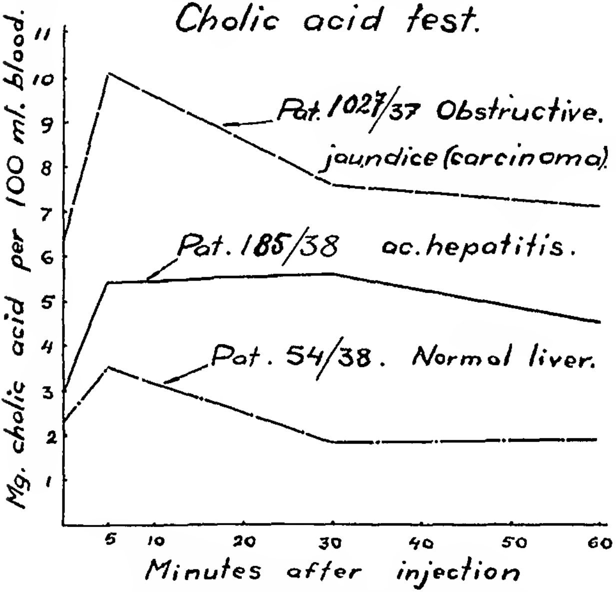


Fig. 4. From figures by Josephson (1939a). Cholic acid tolerance test. Blood concentration of cholic acids before and 5, 30 and 60 minutes after the intravenous injection of 0.50 gram cholic acid in human subjects. The curves represent three typical cases.

the injection and that after 30 minutes of more than 2 mgm. per 100 ml. indicates an obstruction, while a difference of less than 1 mgm. per 100 ml. indicates a parenchymatous disease. These differences are limit values. Usually the difference between the two types of elimination is much more obvious.

Since the publication of this bile salt tolerance test the results have been confirmed in this laboratory by further observations on other cases. Unfortunately, however, the bile salt determination method as described by Josephson (1935) will scarcely have any future in clinical work as the cholate determinations are far too complicated and time-consuming for routine use. For this reason another way of following the cholate elimination has been achieved, the surface-activity of the bile acids being used as a means of following changes in their concentration. The method is very nonspecific and does not give any idea of the real bile salt concentration of the blood. However, the value of this absolute concentration is of very little clinical interest. The surface tension of the blood or serum of one individual is very stable if no surface-active substance is added. A bile salt injection, on the other hand, causes an obvious change in the surface-tension, which returns to the original level when the salt is eliminated from the blood. By studying the rate of this return it seems possible to follow the elimination in a convenient way without obtaining any values of the real bile salt concentration. The method will be published later in full detail.

In cases of liver diseases the formation and circulation of the bile acids seem to be rather independent of other functions of the liver. The blood-cholates are seldom increased and the cholate elimination test always turns out normally when the bile pigment concentration of the blood is normal, but in other respects there is very little parallelism between the amount of bile acids in the blood or bile and the production and concentration of other substances with connection to the liver, e.g., cholesterol, plasma protein, urea, etc. Even the plasma prothrombin seems to be independent of the bile acids in the intestines to a certain degree. In this laboratory it has been repeatedly observed that the prothrombin level may be normal in spite of a poor bile acid production. In fact, the most convenient way in which the clinician may obtain a rough idea of the state of the bile acid circulation is by studying the feces as the fat resorption is very susceptible to disorders of the bile acids in the intestines. The drawing of conclusions concerning the production or circulation of the bile acids from results of investigations of other liver functions may easily give erroneous results.

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# THE PHYSIOLOGY OF THE GENE

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In textbooks of physiology, it is customary to treat the cell as the ultimate living unit. The properties of the cell are interpreted as resulting from the interactions of individually non-living substances when organized in the physical system characteristic of protoplasm. A different treatment is found in genetics. The ultimate unit of life here is not the cell but the gene. The purpose of this paper is to review briefly certain aspects of the rôle of genes in cellular physiology. Its scope forbids any attempt at an exhaustive review of the pertinent literature (cf. 101, 256).

*The Gene as a Unit of Heredity.* The gene concept is based primarily on results from breeding experiments. It is found that most of the differences among individuals of a species can be interpreted successfully as due to combinations of differences in numerous separable components of a postulated hereditary material; and in the environments. A gene, from this standpoint, is one of a number of alternative conditions of a hereditary component, occurring in an individual only, as a rule, if in continuous unchanging existence along one of the ancestral lines; incapable of resolution by any known experiment into separately transmitted subentities, transmissible asexually to all offspring, but sexually only to a certain proportion (usually 50 per cent). The data directly imply the existence of numerous intracellular entities, each capable of synthesizing exact duplicates irrespective of the nature of the rest of the cell or of the organism or of the external environment, and producible only by such synthesis. The differential effect of any pair of alternative genes (alleles) on the characteristics of individuals is constant among all individuals in which the other conditions (genetic and environmental) are the same, however variable, apparently, in intervening generations in which these other conditions have not been controlled. The most important qualification of these principles is that a gene may occasionally undergo an abrupt change (mutation) to an alternative condition, which thereafter persists and multiplies as of the new sort. The likelihood of undergoing such a change may be increased by certain environ-

mental conditions (treatment with ionizing radiations, high temperature, etc.) but not apparently in a directed fashion. Mutation rate is also sometimes increased by particular associated genes (56, 211). Finally it may be noted that genes differ greatly in stability, the rate of mutation in most cases being apparently less than $10^{-5}$ per generation but with much higher rates in the relatively rare class known as unstable genes.

The unitary character of genes may be illustrated by an experiment (196) in which more than 300 successive back crosses of long winged flies of the species Drosophila melanogaster to a pure vestigial winged stock have not modified in any way the character long wing or its frequency (50 per cent) in each generation, although in the 300th generation the flies were only $(\frac{1}{2})^{300}$ long winged by ancestry. The autonomy of genes is especially strikingly illustrated in experiments in which genes from one species have been introduced into another by repeated back crossing to the latter (59, 266).

*The Gene as a Physical Entity.* A great deal has been deduced about the nature of the system of genes without recourse to any other technique than that of breeding, but, while adequate, these deductions take on a greater appearance of reality when it is found that they are exactly paralleled in all respects by the visible behavior of the thousands of individualized granules (chromomeres) distributed along the unbranched, threadlike chromosomes. A single set of the latter is typically present in germ cells, a double set in the somatic cells, but other numbers are found in just those cases in which the breeding results indicate corresponding other numbers of alleles. The number of pairs of chromosomes agrees with the number of linkage systems. Visible deficiencies, duplications, translocations and inversions occur in the chromosomes where the breeding data indicate that such changes have occurred in the linkage systems.

Until recently the breeding technique has given a much more detailed account of the architecture of heredity than has the microscope but since Painter's discovery of the relationship between individual genes and identifiable granules in the giant chromosomes of the salivary glands of Drosophila (192) the stimulatory effects of discoveries in each field on the other have been more nearly equal.

These chromosomes have an aggregate length of 1 to 2 mm., depending on the amount of stretch, or more than 100 times that of the chromosomes seen at metaphase of mitosis. They show some 5000 identifiable bands of chromatin, separated by more extensive, nonstaining material (27, 28). They are believed to be cylindrical bundles containing a

large number of stretched out replications of the two homologous basic chromosomes with corresponding chromomeres associated to form the bands (cf. 193, 170). Particular genes have been demonstrated to be located on or near particular bands by careful observation of the structure near the ends of the loops and bulges found where one homolog has an inversion, duplication or deficiency (covering known genes) relative to the other homolog. The number of bands agrees roughly with estimates of the total number of genes in Drosophila and there may be a one to one relation. In a study of minute deficiencies induced by x-rays, 4 distinguishable genes (yellow, achaete, scute, lethal) were found to be located in what then seemed a broad single (or perhaps double) band (the second from the distal end of the X chromosome (184)). A visible deficiency of half of this band (some 125 m$\mu$) gave viable flies showing the recessive character yellow and achaete. Later observations, however, showed four visible components in this band (81, 27).

There is some ambiguity in the terms used by geneticists. As noted, the breeder, in referring to a gene, means merely a member of a set of alternative *conditions* in the hereditary material. Whether the condition in a particular case is one of addition, of loss of material, or of rearrangement, he does not ordinarily know. In cytogenetics and physiological genetics, however, it becomes desirable to be able to refer to the actual material at a certain locus as it is when affected by a particular allele. The term gene has come to be used also in this sense.

The question whether the genes in this sense are discrete entities or merely regions in a continuum is an old one. Breeding data merely prove that a certain difference behaves as unitary in the tests that have been applied, but there can be no assurance that such a difference may not turn out to be composite by other tests. From this viewpoint, genes are merely regions of the chromosome within which crossing over or other breakage has so far not been observed to occur.

There are a number of possibilities both with respect to the physical and physiological discreteness of the genes (cf. 32, 101).

1. The genes may be physical units, separated from each other by non-genic material within which exchange at crossing over or breakage under x-ray treatment is likely to occur.

2. The genes may be physical units in immediate contact with each other, the breaks occurring only between genes.

3. The genic material of a chromosome may be a continuum, capable of breakage at any point.

If the gene is a physical unit, it may act as a physiological unit, but it is also possible that it may be composite physiologically or that a group of adjacent genes may act together differently than if more widely separated. If the genic material is a continuum, there may be more or less widely spaced centers of physiological activity separated by relatively inert material or there may be overlapping regions that act as physiological units.

The strongest genetic argument for a high degree of physical unity in the genes is probably the fact that crossing over does not produce the mutational changes that would necessarily occur if there were any inequality in the points of exchange (239). Study of breakages near the distal end of the X-chromosome of Drosophila under x-ray treatment indicated that in this case also the points of breakage tended to be repeated exactly (184).

The idea that genes may be separated by nongenic material encounters one difficulty. The essential difference between genic and nongenic material is obviously the capacity of the former and incapacity of the latter to synthesize duplicates. But if the genes are separated by nongenic material the new genes formed when the system duplicates might be expected to fall apart which, of course, is not the case except for crossovers, occurring perhaps about once in a thousand times between adjacent genes. The persistence of an order is known not to be due to specific attraction of adjacent genes, since new orders due to translocation, inversion, duplication or deficiency persist as strongly as the old order, once they have been brought about. Either there must be continuity of the genic material, or nongenic material must immediately collect between new genes, perhaps produced by them, to bind together those which happen to be adjacent. The latter is postulated in Belling's theory of crossing over (18). The connections between genes cannot rest on polarity, since inversions of order of blocks of genes occur (237).

There is considerable correlation between the gene as a block within which crossing over does not occur and the gene as an apparent physiological unit. Multiple alleles in general affect the same characters and frequently seem to differ only in the degree of effect (review 232). On the other hand, there is little or no tendency for genes that are close together in the linkage system to be similar in effect (e.g., yellow and achaete seem to be adjacent genes in Drosophila (184) but one has its principal effect on body color, the other on development of the bristles). In general the effect of replacing one allele by another is as independent of replacement in neighboring loci as in any other parts of the genetic

system. The translocation of a gene, whether type or mutant, to another region usually has no apparent effect on its physiological activity.

These principles are not, however, without exception. It is now clear that the effects of multiple alleles are by no means always merely differences in degree (232) and in some cases the effects appear to be on unrelated characters, e.g., spineless and aristapedia in Drosophila (241).

In some cases, as first shown by Sturtevant (239), the effect of a gene is demonstrably affected by its position in the system (review 61). Thus, chromosome aberrations in Drosophila often result in character changes (especially changes in viability (195), in stability of effect (177, 58, 217, 107) and in dominance (65)) even when there is no change as far as known in the genic material except rearrangement. In many of these cases it is possible to interpret the results as due to occurrence of a mutation or to loss of genic material near the break but enough cases are known in which a restoration of the original arrangement or a return of an apparently affected gene to its normal position has brought about a return to the normal character to make it certain that position effect is real (239, 65, 66, 194, 110, 57). Only one clear case, however, seems to have been demonstrated in plants (41) in spite of numerous translocations. In these position effects there is, in a sense, an overlapping of the genes as physiological units, but this may mean no more than that interactions between immediate gene products are facilitated by proximity (239, 187).

The appearance of the chromosomes when drawn out to maximum lengths (prophase, and resting phase as seen in the salivary glands of diptera) indicate an alternation of different properties in the chromomeres and the connecting strands (129, 192, 27). The most plausible interpretation at present seems to be that the recognizable genes are relatively unbreakable physical and physiological units, probably associated with the chromomeres and bound together by material that is relatively inert physiologically, relatively easily broken and perhaps not even genic material (in the sense of capacity for duplication).

We have referred to attempts to estimate the number of genes. In Drosophila most of the mutations that are observed now turn out to be at least alleles of previously observed mutations. This indicates that the number of loci is not indefinitely great. Estimates based on frequencies of recurrence rest, however, on the hypothesis, known to be incorrect, that all loci mutate equally frequently. Estimates can also be made on the minimum distances between genes in parts of the chromosome in which rates of crossing over are typical, considered in

relation to the total length of the chromosome. Thus crossover frequencies of 0.1 per cent in a typical region of a chromosome about 100 units long would indicate a minimum of 1000 loci in that chromosome. Finally, is the evidence that genes are associated with the chromomeres. The indications are that there are at least several thousand genes in Drosophila though probably less than 10,000 (173, 176, 181, 109, 54, 27, 28, 255). Belling (17) estimated the number of chromomeres in the lily at about 2000. Lindegren and Rumann (153) put the number in Neurospora at less than 300.

Maximum estimates of the size of genes have been based on estimates of the volumes of the chromosomes, divided by minimum estimates of numbers of genes. These lead to maximum estimates (in Drosophila) of about $10^4$ $(m\mu)^3$, approximately the same as for the particles of tobacco mosaic virus and much larger than hemoglobin molecules (about 1 $(m\mu)^3$). It is reasonably certain, however, that the genes constitute only a small fraction of the size of the visible chromosome. A gene may be no larger than a large protein molecule.

The chemistry of the chromosomes and allied questions have recently been reviewed by Gulick (111). Most of the material extractable from sperm heads (consisting largely of chromatin) is thymonucleic acid (about 60 per cent), combined with proteins of a very simple sort (histones and protamines (about 35 per cent)) (142). The characteristic staining reaction of chromatin to basic dyes is a somewhat nonspecific indicator of nucleic acid. The Feulgen reaction is more specific for the pentose component. Caspersson's (37) studies of the absorption spectrum of the salivary glands of Drosophila indicate that nucleic acid (with a strong band at about 2600Å) is in excess in the bands which stain dark in cytologic preparations. Digestion with nuclease removes this material but does not destroy the continuity of the chromosome (168). This is destroyed by digestion in trypsin (37, 168) but not in pepsin (168) in harmony with the hypothesis that continuity resides in the protein constituent and particularly in protamines and histones (not dissolved by pepsin). It is interesting that the crystalline viruses (230) which behave as genic material in the sense that particles are able to bring about the synthesis of more of their own kind of material in living protoplasm (tobacco virus in either tobacco or tomato cells) are nucleoproteins (5).

Nucleic acid is itself too simple a material and too uniform in nature to be responsible for the specificity of the genes. Similar statements have been made with respect to the protamines and histones extracted

from sperms. The possibilities of diversity among protein molecules through the different possible arrangements of the amino acids and through attachment of prosthetic groups is, however, so nearly infinite that there seems to be no theoretical difficulty in connection with gene specificity, even if only a minute portion of the visible chromosome is genic.

*Gene Duplication.* The most essential property of a gene is, of course, the capacity to bring about the production of an exact duplicate, irrespective, within limits, of associated genes or environmental conditions. The growth and fission of a cell present a certain analogy, but as cells are wholes of which the genes are components, it is to be expected that an understanding of cellular growth and reproduction must be based on gene duplication rather than the reverse. Consideration of the maximum possible size of genes and their regularity of duplication leads in the direction of an interpretation in terms of molecular mechanisms and hence to an analogy with crystal growth (141, 1, 176). There is the difference, however, that duplication must be associated with a mechanism of separation.

Among crystals the closest analogy is with the nucleo-proteins responsible for virus infections. As already noted these are of the same order of size as genes and have the same property of autosynthesis. The similarity of genes and viruses in these respects was indeed pointed out (67) before the discovery of the crystalline nature of the latter. More recently it has been shown (108) that viruses resemble genes in inactivation under x-ray treatment and that viruses may acquire new specificities (after x-radiation) analogous to mutations of genes (163).

The autosynthesis of a molecule of the degree of specificity of a protein seems to require, as often noted, that the original molecule behave as a model on which the duplicate is built up from the simpler substances in the medium. This requires attraction of like by like (cf. 182). The essential pattern can hardly be more than two dimensional to permit such duplication to be followed by separation. Such a scheme obviously provides for duplication as of the new type after mutation.

The pairing of homologous chromosomes at zygotene of meiosis and in the salivary gland cells of diptera demonstrates that there actually is attraction of like for like in cells under certain conditions. This attraction moreover has its seat in the chromomeres rather than in the chromosomes as wholes, since the pairing of homologous chromomeres occurs in cells heterozygous with respect to translocations, inversions, etc., even though the chromosomes must be thrown into crosses, loops,

etc., in order that this may occur (16, 161, 192). The suggestion that the postulated process in which genes attract an array of substances from the medium to form a duplicate is physically allied to the visible attraction between homologous chromomeres has often been made.

One of the most important lines of investigation on the nature of genes is the study of the occasional failure of exact persistence or duplication. There is an extensive literature on mutation which has frequently been reviewed (244, 124, 179, 188, 245, 218, 247). Considerations of space forbid such a review here. It may be noted, however, that the conception of the gene that seems to fit best the facts of mutation is that of a molecular pattern which may be replicated but not many times, usually highly stable, but with a threshold for internal rearrangement or other change that is easily surpassed on absorption of energy from a single excitation or ionization by x-rays, $\gamma$-rays, neutrons, etc., and occasionally surpassed by extreme thermal agitation (248).

*Control of Genes by Cellular Physiology.* While genes behave as autonomous entities in their capacity for duplication and in the incapacity of the cell to replace ones which have been lost or inactivated, in other respects they behave as components of an integrated system. For example, all chromosomes and hence presumably all genes duplicate as a rule just once in the cycle of cell division. It would exceed the scope of the present paper to discuss the processes of mitosis and meiosis and their implications for gene physiology.

It should be added, however, that while in these processes the genes behave as if subject to the physiological conditions of the cell as a whole, there is evidence that this physiology is in turn regulated by particular genes. A considerable number of genes are known which affect the regularity of mitosis and meiosis (106, 26, 240, 6, 7, 8, 9, 73).

*Non-mendelian Heredity.* The question as to how far hereditary properties are restricted to the chromosomal genes is one that must be considered briefly (reviews 79, 99, 228). In the first place, it must be noted that there are two groups of organisms—bacteria and blue green algae—in which the occurrence of a definite nucleus, of chromosomes, or of any sort of mitosis is usually denied (cf. however (152)). The existence of groups in which all heredity must apparently be non-mendelian suggests the likelihood of some heredity of this sort in higher organisms. Evidence of such heredity was indeed discovered early by Correns (44, 45).

The usual criterion for distinguishing non-mendelian heredity from mendelian is based on the great disparity in the amounts of cytoplasm

contributed by egg and sperm in contrast with the essential similarity of the nuclei. Thus a difference between reciprocal crosses which persists at least to $F_2$ suggests transmission in the cytoplasm unless it can be associated with a recognized nuclear difference (such as the distribution of the X and Y chromosomes). A difference, manifested in $F_1$ (and not due to sex linkage) implies dependence on a quality of the egg cytoplasm but not necessarily that this quality is carried autonomously by the cytoplasm from generation to generation. It may have been imposed on the cytoplasm, either by the egg nucleus before maturation or by absorption of substances from the mother. Several cases are known in which a character is mendelian but as an indicator of the genotype of the mother, presumably through one of those mechanisms: serosa color of the silk worm embryo (251, 242), lipochrome coloration of eye and body in newly emerged Gammarus (224, 225), ocellus color of the larva of the moth Ephestia (experimentally shown to be affected by the maternal hemolymph (35, 36, 145, 146), localization of extra bristles in the mutation polychaeta of Drosophila funebris (246), viability and fertility in several cases in Drosophila (205) and direction of coiling in the snail, Lymnaea (24, 238, 60).

There seem to be no wholly clear demonstrations in animals of non-mendelian transmission of *individual* differences through the germ cells. Kühn (144) found a slight but clearly transmissible difference between reciprocals on crossing strains of the wasp Habrobracon selected for difference in pigmentation. It is not clear, however, that the parent strains had been made isogenic before the cross. Jollos (137), working with protozoa, has found transmission for a limited number of generations of effects of treatment with chemicals, high temperatures, etc., that suggest cytoplasmic heredity. The same author (138) has described similarly behaving "Dauermodifikationen" as a result of temperature shock in Drosophila. This has been partially confirmed by Plough and Ives (201), who described a tendency toward sporadic appearance of abnormalities of development after such treatment, for several generations, exclusively down the female line.

Goldschmidt (98, 99, 100) has demonstrated cytoplasmic heredity of a number of differences between geographical *races* of Lymantria dispar (cf. however (264)). These include differences in the strength of the tendency to develop in the female direction, in rate of larval development and in larval pattern.

For characters that develop late in ontogeny a difference between reciprocal crosses (if not due to sex linkage), observed only in $F_1$, raises

a presumption of cytoplasmic heredity. There is evidence of this sort from Triton hybrids (121, 122) and merogones (3, 112, 113).

Clear cases of non-mendelian heredity are much more numerous in plants. Most of the cases of individual differences shown to be of this sort are in chlorophyl characters, especially chlorophyl variegations (cases in about 50 species listed by de Haan, (51)). The plastids divide and are transmitted in the embryo sac, rarely in the pollen. It is likely that these are cases of direct transmission of a plastid quality. It should be noted, however, that the plastids are far from being completely autonomous bodies. de Haan lists 73 chlorophyl characters in the corn plant as due to nuclear genes and only 3 as transmitted in a non-mendelian fashion.

The most exhaustively studied case of a non-mendelian individual difference not related to chlorophyl is one of pollen sterility in corn (210).

It is doubtful whether a sharp line can be drawn between non-mendelian heredity and virus infection. A case in Datura stramonium illustrates the difficulty (23). A type called quercina differed from normal in its leaf shape, low vigor and usual pollen sterility. It followed an irregular, apparently non-mendelian course of heredity. At first disease seemed ruled out by failure of transmission on rubbing leaves, or inoculating juice, but it was later found to be transmissible from affected to normal tissue after grafting.

A rather large number of cases have been described in which crosses between different subspecies or species of plants have given persistent differences in reciprocal crosses, where no question of infection in the ordinary sense is involved. Renner (208, 209) for example has shown that plastids whose straight female line of descent is from Oenothera Lamarckiana do not produce normal green pigment in any plants in which they come to be associated with nuclei of certain constitutions (e.g., homozygous Oenothera hookeri). Sirks (227) made crosses between Vicia faba major and V. faba minor. With major as the female parent, a block of linked genes behaved normally in $F_2$ (no differential viability, normal crossing over). In the reciprocal cross, the $F_1$ individuals exhibited effects of the genes of this block derived from the pollen plant (major), but produced no crossover gametes and in $F_2$ no homozygotes for the major chromosome appeared. The results must apparently be attributed to an interaction between the major chromosome and a transmissible property of the minor cytoplasm. Sirks also found differences in stem length and fruit length that were non-mendelian. Wettstein (258, 259) has described crosses between mosses

showing maternal inheritance of morphological characters for many generations. These were more pronounced in intergenoric crosses than in interspecific ones and were not observed in interracial crosses. Non-mendelian results from crosses between species of Epilobium have been studied by several investigators (reviews 79, 99, 228). Michaelis found that effects of disharmony between maternal cytoplasm and paternal genes on corolla length and pollen fertility were not removed by 13 generations of backcrossing. Pollen sterility is a common consequence of species crosses and has been shown in a number of other cases to be transmitted as if dependent on a persistent disharmony of the cytoplasm with the nucleus of the pollen parent.

Thus there can be no doubt that there are qualities of the cytoplasm that are autonomous. The astonishing thing is that cytoplasmic heredity plays such a small rôle. The general principle that reciprocal hybrids are closely similar if not identical was established by the early plant hybridists—Kölreuter, Gärtner, etc. Gärtner (quoted by Mendel, 169) demonstrated in 30 cases that hybrids could be transformed into a type indistinguishable from the pollen parent by 3 to 6 successive pollinations to the latter, indicating that the entire species heredity was transmissible by the pollen. Goodspeed and Clausen (103) obtained some apparently pure Nicotiana sylvestris from tabacum x sylvestris hybrids after only two such pollinations to the latter. In several cases in plants, merogones have been reported as developing into plants of purely paternal type (cf. 79).

*The Genetics of Individual and Species Specificity.* The usual subject matter of studies of heredity is found in the morphological and physiological characteristics of species and of individuals within species. In most of these cases, however, the relation between gene and observed character must of necessity be rather indirect. It is desirable to consider the most direct indicators of the specificity of different protoplasms and these seem to be provided by serological properties, including the reactions to transplantation.

Leo Loeb (158), working with guinea pigs and mice, demonstrated that the reaction to transplanted tissues (normal or tumor) varies with relationship. Autotransplants are readily accepted. Transplants from close relatives encounter a more or less hostile reaction and are usually rejected in the end. The reaction is more severe toward tissues from random animals of the same species and still more severe toward tissues from a foreign species. Little and associates (157, 154, 156) demonstrated that susceptibility or resistance to tumor transplants depends on

multiple mendelian genes. These genes do not, however, determine susceptibility or resistance as such, but a certain specificity, the reaction depending wholly, or almost wholly, on the presence of dominant specificity genes in the graft lacking in the host. The presence of such genes in the host, lacking in the graft, are of little or no importance. Thus in a typical experiment, all individuals of $F_1$ from a cross between different inbred strains are found to accept transplants from either parent strain. On the other hand, both parent strains reject transplants from $F_1$. The great majority of $F_2$ are usually resistant to a tumor from one of the pure strains. The proportion of susceptible could be interpreted as $(3/4)^n$ where n is the number of dominant specificity genes peculiar to the strain in question. By further breeding it was found possible to develop strains giving simple mendelian ratios (cf. 235, 21). Similar results have been obtained with leukemia in mice (162, 212) and with normal tissues in mice (155) and in guinea pigs (160). Results from inbred strains of rats, on the other hand, could only be harmonized on the hypothesis that these strains had not reached the same degree of homozygosis in the pertinent factors as in the other cases (159).

An especially important case is that of the blood groups discovered in man by Landsteiner (review, 262). The major groups (O, A, B, AB) have been shown to depend on a series of 3 multiple alleles, of which two ($I^A$, $I^B$) determine the presence of the specific isoagglutinogens A and B, while the third is recessive to the others and seems to determine nothing positive, at least in relation to A and B (group O = ii, group A = $I^AI^A$ or $I^Ai$, group B = $I^BI^B$ or $I^Bi$, while group AB = $I^AI^B$) (20). Cells from an individual with either A or B agglutinate in serum from an individual lacking the corresponding gene in analogy with the results from transplants. More recently it has been shown that A consists of two closely similar agglutinogens $A_1$ and $A_2$, determined by different alleles.

An independent pair of agglutinogens M and N, requiring absorbed serum from sensitized rabbits for demonstration, has been shown to be determined by a pair of alleles (148). There is no recessive in this series corresponding to i.

A number of such blood groups have been demonstrated in other mammals. Antigens serologically identical with A and B have been found in anthropoid apes. A series of 4 blood groups in rabbits is determined by 3 alleles of which one was recessive to the other two, parallel to the case in man (39, 40). These cases in mammals are

interesting as showing that specificity of the cytoplasm of anucleate cells may be determined by mendelian genes which must have acted before the nucleus was lost. The allelism of genes determining 2 or more antigens which differ positively from each other (as A and B) indicates a direct relation between the specificities of gene and antigen.

Specificity differences in the red blood cells have also been demonstrated in birds. Todd (249, 250) has shown that, in the domestic fowl, individuals can nearly always be distinguished serologically, even among close relatives. A polyvalent serum, capable of agglutinating the corpuscles of any fowl, would usually agglutinate the cells of a chick after absorption by cells of one of the parents but never after absorption by cells of both parents. The results imply that these differences are entirely dependent on multiple mendelian genes.

Especially instructive are the results of Irwin and associates (133, 134, 135, 131, 132) on 4 allied species of birds, the domestic pigeon (Columba livia), a wild pigeon (C. guinea), the ring dove (Streptopelia risoria) and the pearl neck (Spilopelia chinensis). Crosses and backcrosses were available between ring dove and pearl neck, between livia and guinea (reciprocal crosses) and between livia and ring dove, a family cross. The technique used involved production of antiserum from rabbits injected with red blood corpuscles and absorption of this by corpuscles from one or more sources, to produce reagents containing antibodies for the antigens present in the injected corpuscles but not present in the absorbing corpuscles. Each of the 4 species contained antigens lacking in all of the other 3. Other antigens were demonstrated to be common to all 4 species but some were demonstrated to be common to only two or three. The pearl neck was demonstrated by repeated backcrossing to have at least 10 independent dominant antigens lacking in the ring dove and at least 7 of these were isolated to a point at which 1:1 ratios were demonstrated by adequate numbers in the backcrosses. Similarly C. guinea was shown to have at least 6 independent dominant antigens not present in the tame pigeon. The absence of any demonstrable difference due to cytoplasmic heredity was brought out by a study of reciprocal crosses.

One of the most important results was the demonstration that the $F_1$ hybrids between ring dove and pearl neck and those between ring dove and livia do not contain all of the antigens of the parent species but in place of these produce serologically distinct hybrid substances.

A phenomenon of the plant kingdom that has been interpreted as dependent on biochemical specificity is self incompatibility (77, 78).

The commonest genetic mechanism involves a single series of multiple alleles. Pollen tubes with a given allele (e.g., $S_1$) in the haploid nuclei grow too slowly on a style whose cells carry the same allele (e.g., $S_1S_2$, $S_1S_3$, $S_1S_4$ — —) to reach the embryo sac, while there is no inhibition on styles with wholly different alleles ($S_2S_3$, $S_2S_4$, etc.). At least 15 such alleles have been described in Nicotiana alata and N. Sanderae (77) and 37 in Oenothera organensis (83). The specificity differences in fungi required for zygospore formation probably also belong in this category (22, 123).

These cases indicate that protoplasmic specificity is determined simply and directly by genes. A noteworthy point is that, in these higher animals and plants, the evidence indicates that substantially the entire difference in specificity between individuals and species is under control of mendelian genes.

*The Relation of Genes to Growth.* Growth in the sense of increase in the amount of active protoplasm is characterized especially by increase in protein. It has often been pointed out that the number of possible arrangements in even a small protein molecule is practically infinite (cf. 105). The only mechanism by which a given organism can produce particular ones in the array of possible proteins would seem to be autosynthesis by a preëxistent molecule acting as a model. This is the same mechanism that seems required for duplication of genes and suggests the hypothesis that each protein molecule is formed on a model carried in a chromosome (141). This, however, has obvious difficulties. It implies that growth proceeds linearly from minute components of the cell. Moreover the proteins emanating from the nucleus would have to pass through the nuclear membrane to permit cell growth between mitoses. There is abundant indirect and some direct evidence that gene products can pass through the nuclear membrane (review 234). Haemmerling, for example, has demonstrated control of form determining properties of the cytoplasm by the nucleus, by means of regeneration and grafting experiments with species of the giant one-celled alga Acetabularia (116, 117). Such passage seems hardly likely, however, for substances of such high molecular weight as the proteins.

An alternative view is that duplicates or partial duplicates of genes reach the cytoplasm when the nuclear membrane disappears in mitosis and that these can produce duplicates in turn, and so on, permitting exponential increase. But such particles, having the essential property of genes, would give practically pure cytoplasmic heredity unless it be supposed that their genic property is subject to decay. As noted, it

has been shown in some cases that there are cytoplasmic properties transmitted without apparent decay through many generations. These may reside in cytoplasmic proteins which possess the essential genic property (plasmagenes). Unless it is demonstrated that there is ultimate replacement by substances of nuclear origin, it is superfluous to trace them at all to nuclear genes (chromogenes).

There is the difficulty here that if the main constituent of the cytoplasm is autonomous one would expect more abundant evidence of cytoplasmic heredity than is found, particularly in connection with specificity. If the capacity of a protein to produce specific antibodies depends on its structure as a whole it would seem a necessary conclusion that the heredity of specificity is the heredity of protein structure.

It has been shown by Landsteiner (147), however, that the major portion of the specificity of the antibodies produced by a compound protein may be due to relatively simple chemical groupings (haptens), although the latter are unable to produce antibodies by themselves (review, 105). His results indicate that antibodies induced by the combination of a protein with a hapten react specifically with the hapten, either free, or in other combinations, and not to any great extent with the uncombined proteins. It is possible that specificity effects due to haptens whose presence is controlled by particular nuclear genes (chromogenes) overshadow specificity due to protein plasmagenes.

On the physiological side it appears that the presence of a nucleus is necessary for cell growth, although not for cleavage (126). This is not a mere matter of size of the nucleus in relation to cytoplasm. Even small deficiencies are usually lethal in the egg in Drosophila (151). Moreover, it has been shown that the great majority of small deficiencies in the X chromosome of Drosophila are lethal when homozygous, in single epidermal cells in an otherwise normal individual (53, 54, 55). In the presence of the genes of the type known as Minute, crossing over occasionally occurs in somatic cells (233, 139). If the two X chromosomes are properly marked, this crossing over results in recognizable sister spots provided neither chromosome contains a lethal. A cell lethal is demonstrated by the occurrence of an unpaired spot.

It may be concluded that, while the proteins of the cytoplasm are probably autonomous with respect to basic structure, metabolic processes are dependent on active substances, probably of relatively low molecular weight, emanating from the nucleus, and that the specificity of the proteins as antigens largely depends on such substances in combination with them.

*Heredity in Relation to Differentiation.* So far we have treated heredity as manifested in persistence of qualities from generation to generation. In the course of development of any of the higher organisms, the descendants of a single egg come to be exceedingly different along diverse cell lineages. These differences are not due merely to the immediate reactions of essentially similar protoplasms to different local conditions since they persist and are handed on during cell multiplication in a neutral environment. Tumor cells derived from a particular tissue may be transplanted to foreign positions in a long succession of hosts without losing their tissue specificity. Differentiated cells grown in tissue culture may lose more or less of their morphological differentiation but in general may be shown by suitable experiment to retain their physiological differentiation (review 130). This persistence of properties can only be interpreted as involving cell heredity. There are various possible genetic hypotheses.

Weismann postulated a differential sorting out of nuclear determiners in all cell lineages, except those of actual or potential germ plasm. This view does not appear tenable in view of the equational character of mitosis. Every cell receives a full length replica of every one of the chromosomes including, for example, such specialized cells as those of the salivary glands and midgut of diptera (19).

Another possibility is that certain genes are transmitted in an inactivated condition and that irreversible activation is induced systematically under special local conditions. The result would be a developing pattern of heredities related to the spatial organization of the embryo. There is a class of highly mutable genes responsible for certain types of variegation. In certain cases, somatic mutation of this sort is under some degree of local control. Thus in Drosophila virilis (52) the recessive, miniature $\alpha$, mutates freely in both germ line and soma. It gives rise to a mutation, miniature $\gamma$, in which mutation is all but completely suppressed in the germ line. In the presence of certain other genes, mutability in the soma is, however, increased to a point at which all flies are mosaic. If the mutability of a gene of this sort should come under such complete control of local conditions as to mutate in an orderly pattern in the soma, without ever mutating in the germ line, it would obviously give a basis for permanent differentiation in cell heredities.

The usual and most probable view is that cellular differentiation is cytoplasmic and must therefore persist and be transmitted to daughter cells by cytoplasmic heredity. The chief objection is that it ascribes enormous importance in cell lineages to a process which is only rarely responsible for differences between germ cells, at least within a species.

It may be, however, that the more or less complete early isolation of the germ line of higher organisms has come about in evolution to maintain a line of cells with plasmagenes lacking in prosthetic groups and hence in specialized activity but capable in somatic cells of combining with such groups emanating from the nucleus to form molecules that multiply thereafter as plasmagenes of a more specialized sort. Under this viewpoint, differences in the local conditions may bring about differential accumulation of metabolic products arising from the interaction of cytoplasm with nuclear products and environment, and eventually lead to the elaboration of new plasmagenes in the cytoplasm in particular regions of the organism. In fact, in eggs of the less regulatory sort, irrevocable differentiation occurs in certain regions of the cytoplasm before cleavage and so cannot be a consequence of nuclear differentiation of any sort. As development proceeds, each step in the regional differentiation of cytoplasmic heredity increases the diversity of local environments and so facilitates further differentiation.

*Genic Control of Enzyme Differences.* There is a certain similarity in all cells in gross chemical constitution and physical organization. Yet there is the greatest diversity in the substances produced. The usual interpretation is that there is an almost infinite field of possible syntheses of which protoplasm is capable, but that in each case the course of metabolism is guided along particular paths by the particular assemblage of specific catalysts. The demonstration that several enzymes are proteins capable of repeated crystallization without loss of properties has suggested that their specificity rests on the same basis as that of other proteins or protein compounds. The mode of inheritance of enzyme differences is obviously a question of great importance in physiological genetics (cf. 253, 97, 174, 32, 257).

Differences in pigmentation are among the characters of vertebrates whose genetics have been studied most intensively. The melanin pigments are produced in specialized cells which have been shown in several cases (75, 76, 62, 63, 80) to migrate from the neural crest. In certain spotting patterns, it is probable that these cells fail to reach all parts of the skin (cf. 43). In other cases, however, it is clear that the differences in color depend directly on differences in cell physiology. It has been shown from somatic mutations (274, 70, 200) and transplantation (47, 48, 49, 206, 207) that these differences depend largely on genes in the cells themselves rather than on hormones elaborated elsewhere, although the possibility of control by hormones is illustrated by the well known effects of ovarian hormone on plumage color in the fowl.

The melanin pigments are produced from tyrosin or allied substances

(especially dopa) under the influence of oxidases. Cuénot (46), as early as 1903, suggested that genes might control color differences by control of enzymes. Several authors have reported differences in tyrosinase or dopa oxidase content of extracts from skins of laboratory mammals of different genotypes (74, 189, 140, 42). The results were variable and apparently involved factors not under complete control (cf. 204). The situation seems to have been cleared up by the work of Danneel and Schaumann (50) who devised means of separating dopa oxidase from inhibitory substances normally present in skin extracts. They have given evidence for a chain of 3 processes: 1, a process suppressed by x-rays; 2, anoxidative formation of dopa oxidase, occurring in black rabbits (genotype C-) even under continuous high temperature (37°) but only after a period of exposure to temperatures below 33° in Himalayan rabbits, whites which develop black in exposed parts of the skin (219, 221) (genotype $c^hc^h$), and failing completely in true albinos (genotype cc); 3, a reaction resulting in pigment formation, requiring oxygen and inhibited by HCN.

Another method of demonstrating the occurrence of dopa oxidase has been the reaction of pigment cells in frozen sections of skin to buffered solutions of dopa. Differences have been found between different genotypes of the rabbit (220, 143) and the guinea pig (143, 214).

There are mendelian differences in the melanic pigmentation of the beetle, Tenebrio mollitor. Schuurmann (222) found a corresponding difference in ferments from the hemolymph but no demonstrable difference between chromogens extracted from the exoskeleton. The ferments from two genotypes produced different pigments not only with extracts of the natural chromogen but also with pyrocatechin.

Matsumura (166, 167) has described differences between races of silk worms in amylase activity of the digestive juices and of the body fluids as due to two strongly linked pairs of alleles. Strong activity was dominant over weak in both cases.

White color of the fat in rabbits differs from yellow by a single dominant gene (197). The yellow color of the recessive fails if the rabbits are not fed green leaves. It appears that a xanthophyllase is lacking in the recessive.

Dalmatian hounds excrete more nitrogen as uric acid (2 to 3 per cent) than other breeds (0.2 to 0.4 per cent). Low uric acid excretion is dominant over high (190, 252).

Hyper- and hypoglycemia in mice are both recessive to normal (33, 34) and apparently belong to the same allelic series with high blood sugar dominant over low (69).

Alkaptonuria in man has long been known to be a simple recessive condition (96). Alkapton is not broken down in the recessives, presumably because of lack of the appropriate enzyme. The situation appears to be similar (96, 119) with respect to a number of other hereditary errors of metabolism in man, e.g., steatorrhea, hematoporphyria, pentosuria and cystinuria.

A type of hereditary feeblemindedness is associated with excretion of phenylpyruvic acid. This condition appears to be inherited as a simple recessive (198, 199).

There are numerous metabolic differences in plants that depend on genes. An especially interesting case is that of a recessive gene in corn which changes the nature of the starch in the endosperm, pollen grain and embryo sac. Brink found that the effect in the pollen grain, only 2 cell generations from the reduction division, depended on a difference in amylase activity (31).

*Gene Control of Chain Reactions.* Breeding experiments appear rather crude as a method of physiological investigation. Yet from one viewpoint they constitute a rather delicate method, especially if supplemented by study of the physiological and biochemical nature of the character differences. One can make exactly one or two or (in polyploids) more replacements of one of the ultimate physiological units of living protoplasm without experimental disturbance. The quantitative effect of a given gene replacement can often be studied in a great diversity of genetic as well as environmental conditions.

There has been considerable study of the coat colors of mammals from this viewpoint (267, 268, 269, 273, 72), although there is still the disadvantage that the chemical constitution of the melanins has not been worked out. The colors fall into two main series, dark (or melanic) and yellow (or xanthic). Within the dark series it is probable that at least the sepias and browns differ qualitatively. Something of the complexity of the system of interactions may be seen from table 1 which gives the relative quantities of pigment found (by colorimetry or titration with $KMnO_4$) in guinea pigs in combinations of 5 series of alleles (273, 213, 128).

Inspection of the table shows that the various genes do not determine the colors in any simple way. The sepias and browns differ from most of the yellows by the presence of E. There are, however, two ways of getting yellow in the hair in spite of the presence of E. In the combination EA (not shown) there is a yellow subterminal band in each hair, of the same intensity as in the corresponding combination with ee. In Effpp the hair is clear yellow. if colored at all, but a much paler yellow

than with eeffpp. In the former case, it appears that the melanic process is blocked *before* it has interfered with yellow, in the latter case, *after*.

The C series affects the intensity of whatever color is determined by the other genes (C intense, $c^a c^a$ white) but the effects are far from parallel. Only 3 alleles can be disinguished confidently in effect on

TABLE 1

*The average concentrations of pigment in the hair of guinea pigs at birth*

The sepias and browns are given on a scale in which intense black (EPBC) is 100. The yellows are given on a scale in which intense yellow (eFC) is 100. The latter actually has only about 20 per cent as much capacity for reduction of $KMnO_4$ as intense black. Replacement of a, assumed above, by A, replaces the sepia, brown or yellow of E-combinations by the yellow of the corresponding e-combinations in a subterminal band in dark hair. There is no effect in e-combinations. The recessives are here represented by single symbols except in the C— series.

| | SEPIA | | BROWN | | YELLOW | | | EYE COLOR (E, e; F, f WITHOUT EFFECT) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | EFPB EfPB | EFpB | EFPb EfPb | EFpb | EfpB Efpb | eFPB eFPb eFpB eFpb | efPB efPb efpB efpb | PB | Pb | pB pb |
| C— | 100 | 21 | 50 | 17 | 6 | 100 | 36 | Black | Brown | Pink |
| $c^k c^k$ | 90 | 18 | 42 | 15 | 0 | 38 | 5 | Black | Brown | Pink |
| $c^k c^d$ | 82 | 15 | 47 | 13 | 0 | 41 | 5 | Black | Brown | Pink |
| $c^d c^d$ | 64 | 9 | 40 | 11 | 0 | 38 | 5 | Black | Brown | Pink |
| $c^k c^r$ | 94 | 14 | 43 | 13 | 0 | 19 | 0+ | Black | Brown | Pink |
| $c^k c^a$ | 73 | 9 | 37 | 11 | 0 | 18 | 0+ | Black | Brown | Pink |
| $c^d c^r$ | 75 | 7 | 44 | 7 | 0 | 14 | 0+ | Black | Brown | Pink |
| $c^d c^a$ | 40 | 5 | 31 | 6 | 0 | 14 | 0+ | Black | Brown | Pink |
| $c^r c^r$ | 84 | 5 | 45 | 6 | 0 | 0 | 0 | Dark red | Dark brown red | Pink |
| $c^r c^a$ | 46 | 1 | 33 | 1 | 0 | 0 | 0 | Light red | Light brown red | Pink |
| $c^a c^a$ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Pink | Pink | Pink |

yellow (eeF) causing 4 levels (C 100 per cent, $c^{kd}c^{kd}$ 39 per cent, $c^{kd}c^{ra}$ 16 per cent, $c^{ra}c^{ra}$, white, using compound symbols (e.g., $c^{kd}c^{kd}$ includes the 3 genotypes $c^k c^k$, $c^k c^d$, and $c^d c^d$)). In sepias (EPB) these 4 levels are replaced by a series of *waves*—a drop in intensity of sepia accompanying each upward step in yellow except the last. There appear to be differences in *threshold* for sepia and yellow, and *competition* between the two

processes (267). The melanic pigment of the eye (which seems to lack yellow) does not agree with that of the coat ($c^dc^a$ more intense than $c^rc^r$ in eye but less intense in the coat).

Replacement of either P by pp or of B by bb greatly reduces melanic pigmentation in coat, eye and skin, but does not affect yellow. But in the former case, there is exaggerated dilution in the lower compounds of the C-series while in the latter there seems to be a ceiling effect, relatively little difference among compounds of the C-series except in albinism ($c^ac^a$) itself, a situation found also in the mouse (72). The combination of bb with pp produces little if any greater dilution than in Bpp, although there is a somewhat browner quality found in all comparisons of browns with sepias.

Replacement of F by ff greatly reduces yellow. The effect in the lower compounds of the C-series is exaggerated from the standpoint of percentage effect. In $c^{kd}c^{ra}$ all yellow is lost except for occasional traces. Albinism in these combinations behaves as if almost completely dominant over $c^k$ and $c^d$. This replacement (F by ff) has no effect on sepias and browns that carry P but in association with pp all melanic pigment is absent leaving pale yellow or white.

The genes C, E, P and B appear to be completely dominant over their alleles but in the case of F, dominance is not complete, eeFfC having about 94 per cent as much yellow as eeFFC. There is incomplete dominance of various degrees among the lower alleles of the C-series.

Yellow pigment is almost wholly displaced (not merely concealed) by the melanic pigments in most cases where these are present, indicating another sort of competitive effect from that referred to above. In the case of $c^dc^a$ combined with EFppB, however, there is so little sepia that there is often a close approach to pure pale yellow in contrast with the slate color of $c^kc^a$ and $c^rc^r$ in the same combination.

Additional criteria of the modes of action of these genes are given by the dopa reaction (214). That in the hair follicles must be distinguished from that in the basal layer of the epidermis as they do not run parallel. The reaction in both cases is almost as great in yellows (ee) as in the corresponding sepias or browns (E) and does not appear to be affected by the A series. In the hair follicles the compounds of the C-series give reactions closely paralleling in intensity (not in color) the effects on yellow, whether the hair is melanic or xanthic. Replacement of F by ff reduces the intensity of the reaction, while replacement of P by pp or of B by bb has little or no effect. There is an indication here that the dopa reaction in the hair follicles is largely a test for an enzyme

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concerned in yellow pigmentation, even in melanic individuals. The situation is very different in the basal layer of the same individuals. There is little or no reaction unless both C and P are present. Replacement of F by ff seems to have no effect. So far there is parallelism with the effects of these genes on melanic pigment, although a higher threshold in the C-series. Replacement of B by bb, however, seems to be without effect.

It is obvious that the effect of a gene replacement in one genetic background is usually very different from its effect in another. The genes cannot be supposed to be producing pigment independently of each other. The indications are that each acts at one or more definite places in a system of interacting chain reactions and that the effect of each is on the rate of the process which it affects. Figure 10 represents a simple system capable of accounting for these effects.

There has been a great deal of study of the interactions of the numerous mutations affecting eye color in Drosophila melanogaster. Morgan and Bridges (175, 25, 30) showed that while in some cases the combination of two mutations gave effects that appeared to be merely the sum of their separate effects this was by no means always the case. Where mutations resembled each other closely, the combination was also usually indistinguishable. In other cases, they found a disproportionately great effect in the combination, or even a reversal of the apparent direction of effect of one of the mutations. A number of specific modifiers were found, mutations that could be recognized by a modifying effect on another mutant although without apparent effect by themselves.

The relations between brown, scarlet and vermilion illustrate the deductions that are indicated in such cases (271). Scarlet (stst) and vermilion (vv) and the double recessive (ststvv) are all practically indistinguishable. These results suggest that st and v are inactivations of two genes ($st^+$, $v^+$), each of which is solely (or almost solely) responsible for carrying through a different link in the same chain reaction. The result is the same if either or both fail. The fact that the double recessive "scarlet-brown" (ststbwbw) is pure white suggests that bw is an inactivated phase of a gene ($bw^+$) solely responsible for a link in the chain of processes responsible for the pigment left in scarlet. If these deductions are correct both processes should also fail in the double recessive vermilion-brown. This turned out to be practically the case.

Schultz (216) and Mainx (164) found that the pigmentation processes of brown and scarlet not only differ qualitatively but are to some extent

segregated spatially. In scarlet the pigment consists of water soluble, yellowish red granules, largely located in the cells in the outer ends of the ommatidia. In brown the pigment was in highly insoluble, brownish red granules, surrounding the bases of the ommatidia. Mainx compared the effects of more than 20 mutations on these two processes by finding the combinations of each with scarlet and with brown. Two other genes, cinnabar and cardinal, closely resembled scarlet and vermilion and could also be interpreted as inactivations of normal genes determining links in the chain of processes leading to formation of the brownish red basal pigment. Most of the mutations, however, weakened both processes, although to widely different extents in different cases. Only one locus has been found in which the normal allele is so essential that complete inactivation prevents any pigmentation (white eye). Mainx found two loci (clot, sepia) which produced no apparent reduction in the quantity of either basal or distal pigment but instead modified the quality of the latter. He obtained extracts from sepia eyes which contained a ferment (absent in other genotypes) which would not only oxidize the distal pigment of sepia to an insoluble brown but do the same to the pigment of scarlet. This enzyme is interesting as occurring in a recessive mutation.

Another line of attack traces to Sturtevant's observation (236) that vermilion contrasts with other sex-linked mutations in not being autonomous in gynandromorphs. He interpreted this as evidence that the normal allele of vermilion produces a diffusible product.

Beadle and Ephrussi (*12, 13, 84, 88*) found that larval eye disks could be transplanted successfully to the abdomens of other larvae where they developed into slightly distorted eyes. Vermilion and also cinnabar eye rudiments implanted in larvae of red eyed (wild type) stock were found to develop into red eyes, although eyes from some two dozen other mutations developed autonomously in similar experiments. In the reciprocal experiment, the eyes of wild type larvae developed autonomously (red) in vermilion and cinnabar hosts and in all other mutant hosts (except claret where they became claret, an effect not yet cleared up). That the type genes, $v^+$ and $cn^+$, determine the production of a diffusible substance or substances, lacking in vermilion and cinnabar flies, was demonstrated by the induction of increased pigmentation in the nearly white eyes of vermilion brown and cinnabar brown by injecting (243) or feeding (14) the larvae with extracts of pupae from red eyed stock. Various experiments demonstrate further that two substances must be involved and that the production of the $cn^+$-hormone

depends on production of the $v^+$-hormone. Thus vermilion-eye rudiments in a cinnabar host become red, indicating the presence of the $v^+$-hormone in the latter while cinnabar eye rudiments in a vermilion host merely become cinnabar, indicating lack of both substances in vermilion.

The similarity of vermilion and cinnabar (whose type alleles are involved in production of diffusible substances to scarlet and cardinal (whose type alleles act only locally) illustrates the point that diffusible and non-diffusible substances may enter into the same chain of gene controlled reactions (cf. 10).

The analysis of Drosophila eye color is by no means complete but the results already obtained nevertheless illustrate well the possibilities of combined genetic and physiological experimentation (cf. 85, 86, 87, 89, 90).

A similar situation has been found in the flour moth Ephestia kühniella in which a dominant gene A distinguishes a type with black eyes and pigment in brain and testis sheath from a type without this pigmentation (red eyed). Caspari (35) showed that implants of testes from dominants (AA) remain dark themselves and result in blackening of the eyes of aa hosts while aa testes in AA hosts become dark and do not affect the eyes of the host. The effect of the A-substance has been studied in much detail (146, 145).

Another case of this sort has been described in the parasitic wasp Habrobracon juglandii (260, 261). Males are haploid. Occasionally mosaic males appear whose characters are such as to indicate segregation at the first cleavage following failure of the second maturation division. Black-white (Wh-wh) and black-cantaloup (C-c) and black-red (Rd-rd) eye mosaics have a sharp line of cleavage between the colors, indicating the absence of any diffusion. This is not the case in black-orange (O-o) or black-ivory (O-$o^i$) mosaics. A beautiful natural experiment occurred in mosaic males of constitution cO-C$o^i$. The pale colors cantaloup and ivory were separated by a heavy black belt, sharply defined on the side toward cantaloup but fading through orange into the ivory.

Injection and feeding tests have now shown that the A substance of Ephestia is the same (as far as present tests go) as $v^+$ substance of Drosophila and that the O substance of Habrobracon shows a similar relation to $cn^+$ of Drosophila (11). These or similarly acting substances have indeed been found widely distributed among arthropods.

In the cases above, it appears probable that the diffusible substances are relatively directly related to gene action. Gene replacement appears

to affect merely the metabolism and not the morphology of the tissues producing them. There are other cases in which gene replacements make a difference in quantity or quality of a diffusible substance but in which this difference seems to be secondary to morphological effects. This seems to be the situation in the relation of the sex hormones to the X chromosomes of vertebrates (review 263), in the case of a recessive dwarf mouse due to a defective pituitary (229) and in that of the mutation, lethal giant, in Drosophila melanogaster in which delayed pupation is related to defect of the ring gland (114, 115, 215).

Analysis of the system of factors determining melanic pigmentation of the eyes in the amphipod, Gammarus chevreuxi, by Ford and Huxley (94, 95) is interesting from the fact that it was possible to measure the amount of pigmentation at all stages in the process in most of the combinations. The upper limit was reached very rapidly in the presence of gene R, but with rr (temperature constant at 23°) the amount of pigment with various combinations of modifiers (S-M-, S-mm, ssM-, ssmm) increased in sigmoid curves, at widely different rates at first but approaching limits which were all relatively close to the intense black of R although still preserving the same order as the initial rates. With different temperatures, but a constant genotype, a closely similar series of sigmoid curves was obtained. One gene (d) produced a wholly different effect, however. Pigmentation was almost absent until sexual maturity when it rose to the same level as with its allele D in the usual sigmoid course.

The physiology of factor interaction is more advanced in the case of flower colors than in any other case of comparable complexity since the chemical nature of the pigments has been worked out. Genetic studies from this viewpoint were begun by M. W. Onslow (191) and have been continued by numerous workers since (reviews 223, 149).

The colors depend in part on yellow and orange plastid pigments (carotin, xanthophyl) and in part on sap pigments. The two groups are not related chemically and are rarely affected by the same genes. The following discussion will be restricted to the sap pigments.

The anthoxanthins (flavones and flavanols) are responsible by themselves for sap colors ranging from ivory to intense yellow. The anthocyanins give colors ranging from scarlet through magenta and violet to pure blue. They are glycosides of anthocyanidins. The similarity and the essential difference between anthocyanidins and anthoxanthins is illustrated below in the cases of pelargonidin and apigenin respectively.

The yellow flavone luteolin differs from apigenin (ivory) in having

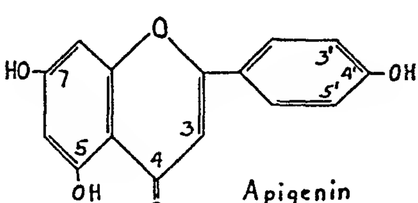
hydroxyls at both 3′ and 4′ on the side phenyl ring. The flavonols differ from the flavones in having the hydrogen at 3 replaced by hydroxyl.

In spite of the similarity of the anthoxanthins and anthocyanidins, there seems to be no such correlation in occurrence as would indicate derivation of one from the other. The evidence indicates rather derivation from a common source.

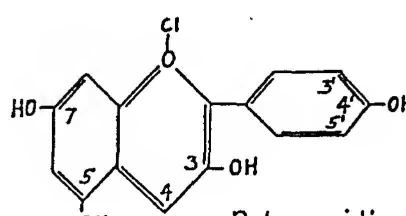
The anthocyanidins fall into three groups according to the number of OH (or $OCH_3$) groups on the phenyl ring. The simplest representatives of these are pelargonidin with one OH (at 4′), cyanidin with two (at 3′ and 4′) and delphinidin with three (at 3′, 4′ and 5′). One or more of the hydroxyls at 3′, 5′ or 7 may be methylated. The actual pigments (anthocyanins) are glycosides. Most of them have a monose (usually glucose, sometimes galactose or a pentose) at 3, a biose (i.e., disaccharide) at 3, or hexose molecules at both 3 and 5.

The color of the pigment depends on a variety of considerations all of which are subject to gene action. The more oxidized pigments are bluer than the less oxidized ones. Methylation tends to redden the color. The same is true of increased acidity. The 3-biosides are bluer than 3-monosides, and 3-5 dimonosides are still bluer. The presence of certain substances including some of the anthoxanthins (e.g., apigenin) tends to change the color of the anthocyanins in the blue direction by what is called co-pigmentation, probably due to weak additive complexes, the derivatives of delphinidin being most affected and those of pelargonidin least. Finally there seem to be special colloidal conditions in some cases, responsible for intense blueness.

An especially instructive analysis is that of Lawrence and Scott-Moncrieff (150) of the colors of Dahlia variabilis. As this is an allooctoploid, it is possible to get each gene in any dosage from 0 to 4, making the



Apigenin



Pelargonidin

case unusually favorable for consideration of the quantitative effects of gene replacement. There are five main color factors. Gene I is necessary for production of the ivory flavone apigenin. Little or none is produced by one dose ($I_1i_3$) indicating a threshold, but deep ivory appears with two or more doses ($I_2i_2$ etc.). One dose of gene Y pro-

duces large quantities of an isomeric yellow flavone. No more is produced with higher dosages. It was shown that this yellow flavone not merely masks the ivory but tends to inhibit its production. An inhibiting factor H tends in turn to suppress the yellow flavone, giving primrose or cream colored flowers and permitting the ivory flavone to develop.

Gene A usually causes the appearance of anthocyanin. Increasing dosage produces increasing intensity leading to deep rosy magenta in $A_4b_4i_4y_4$. The pigment here is entirely cyanin. The addition of apigenin from increasing doses of I has two effects. The color is changed to bluish magenta by co-pigmentation and the intensity is reduced by a competition. Thus $A_1A_3b_4I_4y_4$, is pure ivory. This competition is interpreted as indicating that the two pigments trace to a limited common source.

Gene B is responsible for a much heavier production of anthocyanin than A. ($a_4$ to $A_4$)$B_1b_3i_4y_4$ is a deep crimson purple. The pigment is still all cyanin but with increasing dosages of B, pelargonin appears, shifting the color in the red direction (deep purplish crimson). The addition of apigenin ($I_2i_2$, $I_3i$, $I_4$) again shifts the color in the blue direction by co-pigmentation and again reduces intensity by competition. The tendency to replace cyanin by pelargonin increases.

The yellow flavone has no co-pigmentation effect on the anthocyanins, due either to A or B. It has, however, a more severe competitive effect than apigenin so that even such genotypes as $A_4b_4Y_4$ and $A_1A_3B_1b_3Y_4$ are pure yellow. Such anthocyanin as appears with Y present is wholly pelargonin in the absence of B($A_4b_4Y_1y_3$ deep apricot) unless the yellow flavone is largely inhibited by H, in which case there are mixtures of cyanin and pelargonin (magenta-apricot). It is to be noted that Y shows cumulative effects in combination with A($A_4b_4Y_1y_3$ deep apricot, $A_4b_4Y_3y$ tinged yellow, $A_4b_4Y_4$ clear yellow) although apparently completely dominant in the absence of A or B. A similar cumulative effect is shown in the presence of B. Thus $a_4B_1b_3Y_1y_3$ is scarlet but $a_4B_1b_3Y_3y_1$ tends to be orange and $a_4B_1b_3Y_4$ may be clear yellow.

It is to be noted that no detailed specificity is shown by the anthocyanin factors in this case. Either A or B may produce pure cyanin or pure pelargonin or a mixture depending on other factors. Increase of any pigment (apigenin and yellow flavone as well as anthocyanin) tends to decrease the state of oxidation of such anthocyanin pigment as appears, indicating another sort of competitive effect.

In the China aster (Calistemma chinensis) Wit (265) finds a series of general anthocyanin factors (W, maximum quantity; $w^d$, small quantity;

w, no anthocyanin whatever). The state of oxidation appears at first sight to be determined rather specifically by a series of three alleles. With rr (pink, red) there is pelargonidin glycoside (one OH on phenyl ring), with r′r′ or r′r (lilac, purple) this is replaced by the corresponding cyanidin glycoside (two OH's on the phenyl ring) and with RR, Rr′ or Rr (blue, violet) this is replaced by the corresponding delphinidin glycoside (three OH's on the phenyl ring). These anthocyanins are stated to be somewhat mixed, however, and when ii (assumed above) is replaced by I (incompletely dominant) the colors are darkened and made bluer by addition of cyanidin to pelargonidin in rr and of delphinidin to cyanidin in r′r′ and r′r. It appears that, after all, the R and I series produce a general tendency toward increased oxidation rather than specifically oxidizing particular chromogens.

Several other cases have been analyzed both genetically and chemically with similar results.

We have noted that there is usually a simple one to one relation between gene and the substances responsible for immunological specificity. The suggestion is that these substances or at least the active group (hapten) is a direct product of the gene, perhaps a partial duplicate, reflecting the specificity of the gene itself. A similarly direct relation has been suggested by Mangelsdorf and Fraps (165) in the case of a gene in corn that determines yellow color and vitamin A content in the triploid endosperm. The effect, measured by vitamin A content in feeding tests, was almost exactly proportional to the gene dosage, a relation confirmed by direct determination of the caratinoids (136). In general, however, the relation between gene and such substances as the animal and plant pigments and excretion products is less direct. Many genes, often acting apparently in sequence, are necessary for a particular product. In many cases a gene is more or less replaceable by other genes. Competition effects are common. In certain cases it has been demonstrated that the gene acts on the observed character by determining the presence of more or less specific enzymes. It is likely that the relation of the gene to these enzymes is in some cases of the same sort as the relation to antigens, but interpretation of the observed characters must in any case be in terms of reaction rates determined by such agents.

*Theories of Dominance and Factor Interaction.* Whatever theory is applied to the interactions between different series of alleles should also apply to the effects with a single series. This raises the problem of dominance, including the usual dominance of the normal type over mutations. The first theory was that of Bateson and Punnett (4) who

considered that the dominant member of a pair of alleles is a positive entity of which the recessive is merely the absence. They held that one dose of such an entity was likely to produce as much effect as two on the ground that the total effect might usually be expected to be limited by other conditions. This theory encountered difficulty with multiple alleles and with reverse mutation. If, however, it is supposed that the recessive represents inactivation of the gene in the respect in question, without loss of its essential structure, the latter difficulty disappears. Another theory is that of Fisher (91, 92, 93) that dominance is purely a matter of natural selection. The heterozygotes of unfavorable mutations are held to be selected for similarity to the type of the species until they come to resemble it completely through the fixation of specific modifiers of the heterozygotes. The adequacy of the theory in this form has been questioned (270, 272, 118, 120, 125). Plunkett (202) and Muller (178), however, suggested a selection process tending to build up a factor of safety for the type character in relation to factors of all sorts, environmental as well as genetic. This gives a broader basis for selection and there seems no doubt that such a factor of safety actually is the rule. Whatever the system of modifiers built up by natural selection, there must also, however, always be a complete physiological interpretation of dominance.

There is a certain amount of direct evidence for the inactivation theory of recessiveness. The effect of a deficiency usually resembles or exaggerates the effect of recessives whose locus is in the deficient region (171). This applies in the rare case of a deficiency that can be obtained homozygous (e.g., yellow achaete of Drosophila melanogaster (184)), as well as in the numerous cases of pseudodominance of recessives in heterozygous deficiencies. The dominant allele is usually dominant over deficiencies. These demonstrate that if either the dominant or recessive is inactive, it must be the latter. More satisfactory evidence is given in cases in which three or more representatives of a recessive gene can be introduced into a predominantly diploid zygote. If the recessive has a positive effect causing deviation from type, three should produce more deviation than two, but if it merely has a weaker effect of the same nature as that of the type allele, three recessives should approach closer than two to the effect of the latter. If it is completely inactive, three should have the same effect as two. It appears from such tests as have been made (231, 183, 178) that the completely recessive mutations are largely amorphs (no effect) or hypomorphs (weaker effect than type) in Muller's convenient terminology. Mutations exhibiting partial dominance over type are, on the other hand, usually

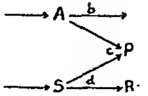
neomorphs (new type of effect) or antimorphs (inhibition of effect of type gene). A stronger effect than type Muller calls a hypermorph.

The majority of multiple allelic series have effects that fall at least approximately in a single linear series, usually with dominance of the highest member over all others, but incomplete dominance in lower compounds. Such series can be interpreted readily as ones in which all variations of the gene, whatever their nature, affect the *strength* of a single primary reaction (268, 272, 172, 180). Such alleles can be classified as amorphs, hypomorphs, type, and hypermorphs.

On the other hand, there can be no question now that there are many multiple allelic series in which alleles have qualitatively different effects. Examples have already been given in connection with serological specificity. Among other cases are the agouti series in mice (68), the black extension series in rabbits (38, 203), brachyury in mice (71, 73), certain colors of the pigeon (127), color blindness in man (254), scute, truncate, spineless and other series in *Drosophila* (64, 29), pericarp color in corn (2, 82), leaf shape in cotton (226) and perhaps certain pattern factors in grouse locusts (185, 186) and in the fish Platypoecilus (15, 104) if these are not due merely to close linkage.

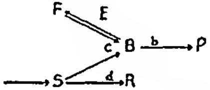
It is indeed probable that multiple changes at a locus are in general all qualitatively different with respect to the structure of the genes themselves and that this is manifested in the effects, if these are directly related to the constitution of the gene, as is probable in immunological properties. Whether manifested in later reactions or not depends, however, on whether the gene or its primary product enters into only one or more than one such reaction (268).

It is usually easy to suggest a plausible interpretation of the dominance relations or the factor interactions in any particular case, but it is difficult to appreciate the implications throughout an extensive network of reactions unless one introduces numbers. For processes such as pigmentation, that take place continuously for considerable periods of time, the simplest hypothesis is that of flux equilibrium for the duration of the process (272, 273). The relation between the rate of formation of a substrate, acted on by agents in various quantities or efficiencies, and the rate of formation of a product is what is needed in attempting to interpret chain reactions under this viewpoint. The rate of formation of the product, $\dot{P}$, depends jointly on the concentrations of the agent, (A), and of the substrate (S), i.e., $\dot{P} = c(A)(S)$. Under flux equilibrium these concentrations depend on the balance between the rates of production ($\dot{A}$ and $\dot{S}$ respectively) and rates of dissipation in the formation of the product (rate $c(A)(S)$), or otherwise (rates $b(A)$ and $d(S)$).




$$\dot{A} - c(A)(S) - b(A) = 0$$
$$\dot{S} - c(A)(S) - d(S) = 0$$
$$\dot{P} = \frac{c}{bd}(\dot{S} - \dot{P})(\dot{A} - \dot{P})$$

If the agent is a catalyst, produced in certain quantity (E) and forming an intermediate product (B) with the substrate, the equation is the same as above or substituting b(E) for $\dot{A}$, where $\dot{P} = b(B) = c[(E) - (B)]\,(S)$.




The quantity E may or may not be the same for all alleles. They may differ independently in the rate constants c and b. The products may differ in specificity in the same sense as the alleles. Thus products formed equally freely from the substrate (same c and b) may differ in efficiency in relation to the characters actually observed. There is no difficulty on this basis in understanding how certain alleles may be amorphs ($c = 0$), others hypomorphs, other hypermorphs in relation to type, and others antimorphs, the last transforming the substrate into a product of zero efficiency and thus destroying substrate that might otherwise be utilized. An allele that produces freely a product of low efficiency would behave as a hypomorph in heterozygotes with an amorph, but as an antimorph in heterozygotes with a more efficient allele. The term mixomorph has been suggested (273). A possible example is found in the genes for white (w), pearl ($w^p$) and eosin ($w^e$) in Drosophila melanogaster (102). The order of effect in females is $w^ew^e$, $w^ew$, $w^ew^p$, $w^pw^p$, $w^pw$, ww. Treating w as an amorph, $w^p$ appears to be a hypomorph in $w^pw^p$ and $w^pw$ but acts as an antimorph in $w^ew^p$ since this is lighter than $w^ew$.

Figures 1 to 3 are intended to bring out the relations in a system in which the rate of formation of a product depends jointly on the concentration of a substrate and of an enzyme, during a period in which the former is in flux equilibrium. This is a limiting case in which b is indefinitely large. At least 3 sorts of curves are of interest in this (and other) cases. In figure 1 the product is plotted against concentration of

enzyme (assumed to be determined by a series of alleles). There is approach to the substrate limit according to the relation $\dot{P} = \dot{S}\frac{c(E)}{c(E)+d}$. There is increasing dominance of genes determining higher values of (E). Curves of somewhat this character have been described for vestigial alleles (172) and white alleles (180) in Drosophila and other cases. A type of curve that is of interest in relation to factor interactions is that in which product is plotted against substrate for different dosages of the agent (fig. 2). In the limiting case treated here, each value of the latter has a certain multiplicative effect on the product produced from a given amount of substrate, in agreement with the common cumulative effect of independent series of alleles. A third type of curve is that of product plotted against time, for varying dosages

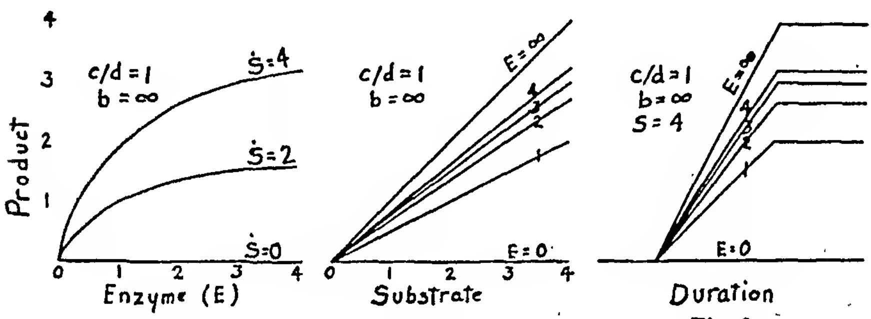



Fig. 1 Fig. 2 Fig. 3

Figs. 1–3. Quantitative relations in a system in which an observed product depends jointly on amounts of substrate and of a gene controlled enzyme, assuming flux equilibrium. Further description in text.

of the gene controlled agent. The simple form of theory used here gives a linear increase during the reaction. The condition of flux equilibrium cannot however hold near the beginning and end of the reaction. Sigmoid curves such as those described by Ford and Huxley (94, 95) are expected.

Figures 4 to 6 illustrate relations between product and substrate in a chain of reactions, subject to a gene controlled agent (A). Figures 4 and 5 represent cases in which c/bd has intermediate values while figure 6 represents the opposite extreme from figures 1 to 3, that in which the product is determined by substrate or agent, whichever is more limited in amount. By interchanging S and A, the curves may also be used to illustrate relations between product and agent for different substrates. A may represent either rate of formation of a noncatalytic factor in the reaction or (if b = 1) the concentration of a catalyst. Curves of the

intermediate type are perhaps more typical of multiple allelic series that act on single characters than either extreme. The ceiling effect, exhibited in extreme form in figure 6, seems to be illustrated by the effects of the albino series in brown guinea pigs (Pbb) as compared with the dark eyed sepias (PB) and even these seem to exhibit considerable ceiling effect compared with the pale browns (ppbb) and pale sepias (ppB).

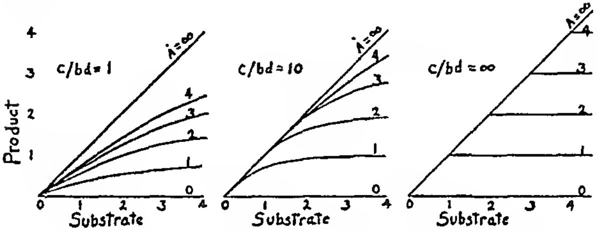



Fig. 4 Fig. 5 Fig. 6

**Figs. 4–6. Quantitative relations between substrate and product for different values of the function of rate constants c/bd and different rates (Å) of production of a gene controlled agent. Further description in text.**

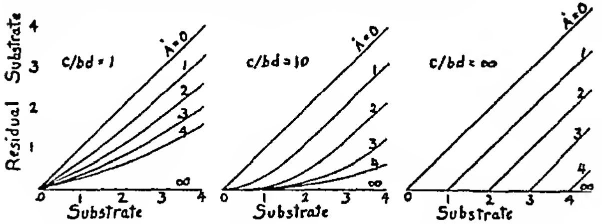



Fig. 7 Fig. 8 Fig. 9

**Figs. 7–9. Quantitative relations between rates of production of substrate and of a product other than that due to the gene controlled agent under consideration, which thus behaves as an inhibitor. Further description in text.**

Figures 7 to 9 illustrate the relations between rates of production of substrate and of a product (R) other than that due to the agent under consideration. Genes determining the latter thus act as inhibitors of the product in question. Figure 9 represents the extreme case in which increase in the agent has a simple subtractive effect, and incidentally a threshold effect, on the product as related to the substrate. Several

examples of threshold effects may be found in the data in table 1. Figures 7 and 8 illustrate cases intermediate between the subtractive effect (found if $c/bd = \infty$) and the multiplicative reduction of the product (found if $c/bd = 0$).

Figure 10 represents a possible system of relations among the color factors of the guinea pig in which the insertion of suitable rate constants according to the above theory gives reasonably good agreement with

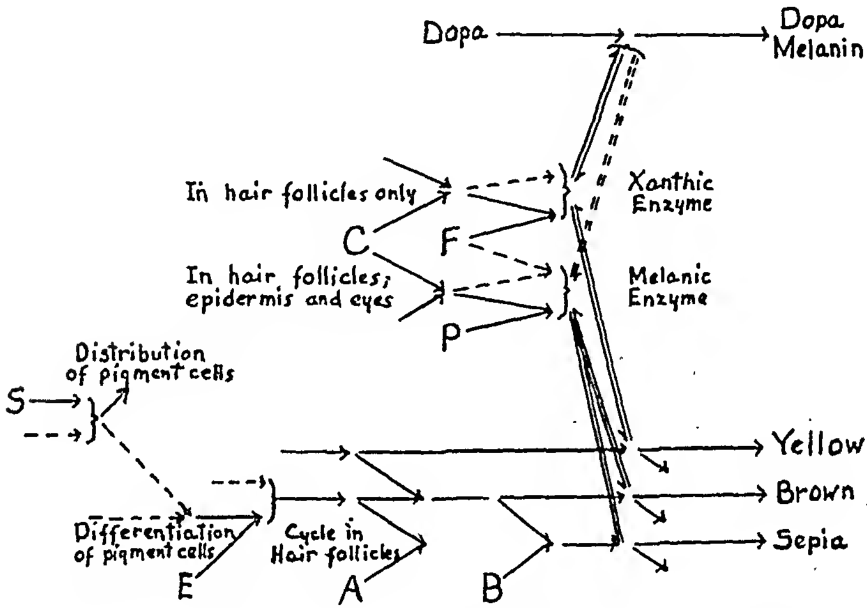



Fig. 10. A quantitatively possible interpretation of the relations between the actions of certain genes of the guinea pig and the production of melanin pigment. The gene s in place of S (not discussed in the text) produces a pattern of colored spots on a white ground, probably by restricting the distribution of melanophores. It also reduces the amount of melanic (as opposed to xanthic) differentiation in tortoise shells ($e^pe^p$).

the observed quantitative relations in all factor combinations as well as accounting for the dopa reactions. While it is not the only possible scheme, the data impose severe restrictions on hypotheses. There seems little doubt that each gene or its immediate product has a modifying effect on reaction rates at one or more points in a system of chain reactions of this general nature.

*Morphogenesis.* Among the most interesting problems in physiological genetics is that of the control of developmental patterns by a

system of genes, believed to be distributed without change to every cell in the body. A possible mechanism of such control has been suggested in connection with the discussion of cytoplasmic differentiation. The scope of the present article does not permit review of the extensive literature bearing on morphogenetic action of genes.

In general, the reaction chains connecting primary gene action and observed effects on morphological characters must be longer, more ramifying and more heterogeneous than where effects are on intracellular products. Even more indirect are the relations of genes to modes of behavior of the organism as a whole, although there are cases in which there is simple mendelian heredity. Most indirect of all is genetic control of extraorganic structure (form of spider webs, nests of termites, of wasps, of birds, etc.). The heredity of structures at this level is controlled by hereditary behavior of the organism which is controlled by hereditary structure at the organic level, which in turn is controlled by hereditary cellular behavior and this by elements of cellular constitution tracing perhaps directly to gene products.

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# FACTORS AFFECTING THE TESTS OF KIDNEY FUNCTION

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Tests for renal function as generally used are of three types. These are: the clearance method which includes the renal clearance of any substance which is in the blood and is excreted in the urine; the rate of excretion of some substance which is administered, such as phenolsulphonephthalein; and the determination of the maximum and minimum specific gravity of urine. This paper is a discussion of the various factors which may modify the first and last mentioned tests of renal function, almost exclusively from the viewpoint of the physiology of the kidney.

UREA CLEARANCE. *Nervous effects.* The question of nervous control or modification of urea clearance has stimulated many experimental studies which have been cited by Rhoads and co-workers (1) and by Smith (2). The latter has pointed out that anesthetics complicated the studies of most workers. Rhoads and associates found that novocainization or total section of the nerves in the renal pedicle of dogs with a kidney explanted under the skin had no effect upon urea clearance. Inasmuch as the dogs had been unilaterally nephrectomized renal hypertrophy probably had occurred. This could hardly be the cause of their negative results because the clearance could be greatly changed by means of diet. Page and Heuer (3) have reported that denervation in one patient with essential hypertension did not change the urea clearance. Freyberg and Peet (4) have reported the effect of bilateral splanchnicectomy upon urea clearance, maximum specific gravity and blood pressure in a series of 48 cases of hypertension. They considered that the hypertension was greatly relieved in some, but that there was no effect in others. In general they thought the changes in the kidneys were associated with the changes in blood pressure. In those patients who had a significant and maintained decrease in blood pressure, urinary abnormalities decreased or disappeared and the renal

function, if it had been impaired, improved. When hypertension was lowered in patients having normal renal function, the efficiency of the kidneys remained normal. When the hypertension was not favorably influenced, renal function remained unchanged or gradually became worse. Inspection of the data presented indicated to the writer that the operation resulted in 24 of 44 cases in no material change in urea clearance, in 9 the clearance decreased and in 11 there was a significant increase. In the latter group systolic pressure pre-operatively ranged around 225 to 260 mm. Hg and was markedly lowered by the operation.

The concentration of blood urea which might be expected to modify urea clearance was found by Addis and Drury (5) to have no effect upon the Addis urea ratio. This value when multiplied by 0.6 is that of urea clearance. In human subjects the blood urea changed over five-fold and in rabbits it was elevated from 27 to 1305 mgm. per cent with no change in the urea ratio. Möller, McIntosh and Van Slyke (6) reported that the administration of urea to normal men was without effect upon urea clearance. Van Slyke, Rhoads, Hiller and Alving (7) reported that in dogs with explanted kidneys the urea clearance and the proportion of urea removed from the blood by the kidneys was independent of the concentration of blood urea. At variance with these results are two recent reports. Fowweather (8) reported that after the administration of 15 grams of urea to each of 50 medical students who had no history or evidence of renal disease, the urea clearance data fell into a narrower range and were within the normal range, whereas before urea ingestion the results varied widely and many clearances were unexplainably below normal. However, the results of similar studies upon 20 patients, some of whom had nephritis, did not support Fowweather's claim. Morton and Mussey (9) reported confirmation of this observation on 50 normal individuals. The abnormally low clearance disappeared after ingestion of urea. In 15 cases of nephritis these workers considered that in mild nephritis the clearance increased and with more severe nephritis decreased after the ingestion of urea. Their data upon the nephritics seem too variable to permit generalization. The explanation for these two opposing results of urea ingestion is not evident. The diuretic action of urea by increasing urine volume might have influenced the clearances obtained by the latter workers. They did not report the urine flow before and after urea ingestion.

Another constituent of the blood affecting urea clearance is hemoglobin. Brown (10) in a study of urea clearance in pregnancy reported one case in which an increase in hemoglobin from 25 per cent to 50 per

cent was accompanied by a rise in clearance from 18 per cent to 66 per cent of normal. Fouts and Helmer (11) extensively studied urea clearance in cases of pernicious anemia. They concluded that there may be a marked increase in the clearance of such patients after a remission induced by liver extract. This occurred in 17 of 50 cases. They considered that the patient, having the complicating factors usually associated with an increased requirement of orally administered liver extract, was likely to show a low value for urea clearance even after the red cell count had reached normal. Also, the patient with a low clearance was more likely to require liver extract by injection to maintain a normal red cell count than the patient with a normal clearance. Of course the relapse and remission of pernicious anemia may affect the kidney directly as well as by the level of hemoglobin.

Diet has a marked effect upon urea clearance especially in dogs. Addis and Drury (12) reported that the urea ratio was increased in the human subject 3 to 5 hours after ingestion of a mixed meal, glutamic acid or milk. Cane sugar or alcohol had no apparent effect. Jolliffe and Smith (13) found that in dogs the urea clearance was reduced under the conditions of fasting or a low protein diet, but was greatly increased by a meat diet. The effect of the meat feeding persisted 16 to 18 hours after the last meal. This effect of meat feeding in dogs was confirmed by Van Slyke and associates (14). Pitts (15) found that meat, casein, glycine and alanine increased urea clearance in dogs. However, the protein intake in the human subject has less effect upon urea clearance. Cope (16) reported that raising the protein content of the diet of hospital patients from 75 to 120 grams was without consistent effect upon urea clearance. On the other hand, lowering protein intake from 75 to 40 grams was accompanied by depression of the clearance in 11 of 12 subjects with normal or nearly normal renal function. In 3 patients whose clearance was below half the normal, the low protein diet had little effect. Goldring and associates (17) reported that the urea clearance at urine flows above the augmentation limit in normal men subsisting on diets containing 9 or 280 grams of protein per day, was reduced about 23 per cent at the lower protein level but showed no elevation at the higher protein level as compared with a control period when 100 grams of protein per day was given. Farr (18) found in 4 children, aged 4 years, with the nephrotic syndrome, that the urea clearance varied markedly with the protein intake. Protein intakes of 0.5, 1, 2, 3 and 4 grams per kilo per day were accompanied by average urea clearances of 73, 88, 178, 184 and 216 per cent of mean normal,

respectively. Administration, during low protein periods, of urea sufficient to produce urea outputs like those observed during high protein intake caused relatively slight increase in urea clearance.

The vitamin A intake has been demonstrated by Herrin and Nicholes (19) to have an effect upon the magnitude of urea clearance. Avitaminosis A in dogs resulted in urea clearances which were about 70 per cent of normal and administration of large doses of vitamin A to these deficient dogs and normal ones resulted after a few weeks in clearances markedly above normal. The variations in urea clearance were effected by parallel changes in the rate of glomerular filtration and were due to changes in function and not to anatomical changes in the kidney. The administration of large doses of vitamin A to normal human subjects receiving their usual mixed diet produced variable results. In 2 of 17 subjects the urea clearance did not change, in 14 the clearance increased 11 to 15 per cent and in 7 the clearance elevation ranged from 24 to 91 per cent. The subjects in the latter group seemed to be the type who easily gain in body weight (20).

Variations in the intake of NaCl from minimal to large amounts was found by Cope (16) to have no effect upon urea clearance in human subjects. In one instance as much as 65 grams were ingested and excreted in the urine at high concentration with no effect upon clearance. Landis, Elsom, Bott and Shiels (21) extensively reviewed the literature and studied the effect of NaCl restriction upon urea clearance in patients with renal insufficiency. They used 12 hour or 24 hour clearance periods because they found less variation in the data than that obtained with a one hour period. With the diet and fluid intake maintained constant, a restricted intake of NaCl was accompanied by a slight elevation of plasma urea and slight diminution in the 24 hour urea clearance. Administration of NaCl reversed these effects. In one patient with advanced renal insufficiency plasma urea decreased from 154 to 26 mgm. per cent during NaCl administration. Acute restriction of NaCl produced hypochloremia with characteristic symptoms, and temporary retention of urea, creatinine and phosphate. Administration of NaCl relieved symptoms and diminished the blood content of urea, creatinine and phosphate. In this patient urea clearance over 24 hours was slightly lower during and shortly after NaCl restriction, and was higher during NaCl administration. The effects of lack of NaCl appeared only after several days due, they thought, to the time required for depletion of body stores of Na.

*Effect of age.* The urea clearance of 9 normal infants, according to

Schoenthal, Lurie and Kelly (22), when corrected for surface area, agreed with values established by Van Slyke for adults. The average urea clearance in children was considered by Payne and Shukry (23) to be higher than in adults. In 39 children free of renal tract disease, the average was 80 to 140 per cent with only 8 cases below 80 per cent.

Cullen, Nelson and Holmes (24) determined urea clearance in 62 children with no evidence or history of renal disease. The data were analyzed biometrically. They concluded that the distribution and mean values of the urea clearances corresponded to those of normal adults. The ages ranged from 5 to 13 years and there was no significant difference in clearance with age or sex. Lewis and Alving (25) measured urea clearance in 100 normal men, 40 to 89 years of age, twenty in each decade and in 2 men 91 years and one man 101 years of age. The clearance declined with age. Their equation for the decline in the men aged 40 to 89 years is, urea clearance in per cent of clearance of normal young men, = 136.6 − 0.912 x age in years. The decline over this 50 year period was 46 per cent. The mean values in succeeding half-decades or decades suggested to these workers, that the decline does not follow a straight line regression, but an S-shaped curve, being arrested from 55 to 65 years and accelerated from 65 to 75 years. Furthermore, the blood urea in this same group over the 50 year period increased 46 per cent. The gradual increase of blood urea followed sometime after the decrease of urea clearance.

*Muscular activity.* Addis and Drury (5) found that the urea ratio decreased markedly in a human subject during an hour of strenuous exercise but was at a normal level during the first and second hours after the exercise. MacKay (26) noted a similar effect of exercise upon the urea ratio. Van Slyke, Alving and Rose (27) found that in normal subjects, or in nephritics with more than 50 per cent of normal blood urea clearance the clearance was not materially different, whether the subjects were kept in bed during the determination or were walking about. In 3 of 12 nephritics, with less than 50 per cent of normal urea clearance, the mean clearances observed with the subjects in bed, rising and walking about were 44, 66 and 67 per cent respectively of the clearance observed in the same patients during bed rest. Severe exercise taken by 3 subjects with normal urea clearance depressed the clearance somewhat but in only 3 out of 22 clearances determined during heavy exertion were the values below 70 per cent of the mean normal. Light and Warren (28) determined the effect of playing the regulation games of football, soccer and basketball by boys 14 to 20 years of age, upon

their urea clearance and the protein content of urine. A definite fall in clearance occurred in every one of the 29 boys during the period of the game. The clearances during the games ranged from 47.5 to 37.3 per cent of the subject's normal. There was no correlation between the decrease in clearance and degree of albuminuria.

*Effect of pregnancy.* Brown (10) in 1938 extensively surveyed the literature. The clearance in normal pregnancy was found to be markedly elevated by Cadden and McLane, Hurwitz and Ohler, and Nice but was decreased in the reports of Cantarow and Ricchiuti, and Dieckmann (29). In Brown's series of 41 cases of normal pregnancy, of 54 clearances, only 2 were below 100 per cent and these occurred in the same patient. The average urea clearance was 134 per cent with a range of 78 to 195. The clearance did not change with increase in the gestation period contrary to the decrease presented in an earlier report. Age and parity did not have any influence upon the urea clearance.

Brown concluded on the basis of 196 clearances upon 170 cases of toxemia of pregnancy that the urea clearance was of considerable value in the differential diagnosis between pre-eclamptic toxemia, chronic nephritis and essential hypertension. Before delivery a high value excludes chronic nephritis and if the blood pressure remains high with little other evidence of toxemia and the clearance is high, the case is probably one of essential hypertension. If the clearance is low the result is of no differential diagnostic value in pregnancy but the clearance after delivery is significant, in that most patients with pre-eclamptic toxemia show a rapid rise within a week or two of delivery while in chronic nephritis the percentage use is much less and the clearance never reaches a normal level. The follow-up of patients after delivery showed that if the clearance is high during the toxemia the prognosis is good even if the degree of toxemia is clinically severe. Chesley (30) has reported a study of renal function in 599 cases of toxemia and 119 of normal pregnancy. He concluded that the urea clearance is about the same in normal pregnancy as out of pregnancy, and that in eclampsia, pre-eclampsia and hypertension the urea clearance does not differ significantly from values observed in normal patients. This striking divergence of Chesley's conclusions of the effect of normal and abnormal pregnancy upon urea clearance from those in the literature is not comprehensible. He has quite justifiably criticised some of the reports of low clearances in the toxemias of pregnancy on the basis that the urine flows were extremely low. Generally the reports give no actual urine flows but most of the workers calculated either standard or maximum

urea clearances. The excellent paper of Nice presents conclusive evidence that in normal pregnancy the urea clearance is higher than when the same individual is non-pregnant. Inasmuch as she determined clearances from the fifth month of gestation to the eighth month post-partum, her data are far superior to a single urea clearance which is merely referred to a standard. This method of using the urea clearance is a control for the tremendous variation in urea clearance from one individual to another. For this same reason the report of Brown that the clearance in cases of toxemia rapidly rises after delivery is particularly significant.

*Influence of hormones.* The subcutaneous injection of adrenalin in the rabbit was found by Addis, Barnett and Shevky (31) to be followed by an increase in the urea ratio. With large doses a decrease occurred. The subcutaneous injection of pituitrin was followed in all effective amounts by a decrease in the urea ratio (32). Addis and Drury (12) reported that a large dose of adrenalin injected intravenously in a human subject greatly reduced the urea ratio. Pituitrin injected subcutaneously markedly reduced the ratio in 2 subjects. With both drugs the reduction in clearance was paralleled by a very great diminution in urine flow. Farr, Hare and Phillip (33) produced a persistent polyuria in cats by surgical intervention of the hypothalamico-hypophyseal complex. They found no significant difference between the urea clearance of normal cats and that of cats with diabetes insipidus. Pitressin acted on cats with this type of diabetes insipidus to reduce urine volume but increased their urea clearance as well as the clearance of normal cats. Anterior pituitary substance increased the urea clearance of both normal cats and those with diabetes insipidus. This effect in the latter animals was not solely due to increased urine volumes. White and Heinbecker (34) produced experimental diabetes insipidus in dogs by surgical interference with the hypothalmico-hypophyseal system. They found the creatinine clearance unchanged during the transitory phase of experimental diabetes insipidus and the urea clearance somewhat elevated probably because of the increased urine flow. Creatinine clearance in the dog measures glomerular filtration. Both clearances were normal during the normal interphase. Soon after onset of permanent polyuria both clearances gradually fell. Creatinine clearances reached a level about half of normal by 2 to 4 weeks after onset of permanent polyuria. Urea clearance fell less than the creatinine during permanent polyuria and in some cases did not fall at all. Under the influence of anterior lobe administration, creatinine clearance rose slightly, the urea clear-

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Brown concluded on the basis of 196 clearances upon 170 cases of toxemia of pregnancy that the urea clearance was of considerable value in the differential diagnosis between pre-eclamptic toxemia, chronic nephritis and essential hypertension. Before delivery a high value excludes chronic nephritis and if the blood pressure remains high with little other evidence of toxemia and the clearance is high, the case is probably one of essential hypertension. If the clearance is low the result is of no differential diagnostic value in pregnancy but the clearance after delivery is significant, in that most patients with pre-eclamptic toxemia show a rapid rise within a week or two of delivery while in chronic nephritis the percentage use is much less and the clearance never reaches a normal level. The follow-up of patients after delivery showed that if the clearance is high during the toxemia the prognosis is good even if the degree of toxemia is clinically severe. Chesley (30) has reported a study of renal function in 599 cases of toxemia and 119 of normal pregnancy. He concluded that the urea clearance is about the same in normal pregnancy as out of pregnancy, and that in eclampsia, pre-eclampsia and hypertension the urea clearance does not differ significantly from values observed in normal patients. This striking divergence of Chesley's conclusions of the effect of normal and abnormal pregnancy upon urea clearance from those in the literature is not comprehensible. He has quite justifiably criticised some of the reports of low clearances in the toxemias of pregnancy on the basis that the urine flows were extremely low. Generally the reports give no actual urine flows but most of the workers calculated either standard or maximum

ciates (7) to be followed by an average increase of 43 per cent in urea clearance of the remaining kidney. The blood flow was increased 68 per cent and oxygen consumption 81 per cent. The maximum increase occurred about one month after the nephrectomy.

*Effect of temperature and bodily infections.* Goldring (40a) has cited the literature and reported that in the febrile stage of acute rheumatic infection, urea clearance values are usually higher than the highest observed normal and during the afebrile convalescent period the clearances are usually lower than the lowest observed normal. In 13 patients he (40b) found the urea clearance increased as a rule during the acute stage of penumonia and was slightly to moderately depressed during the convalescent stage. Page (41) has cited the contradictory literature regarding the observed effects of renal diathermy. He administered diathermy treatment for the period of an hour to 14 subjects that included normal subjects and patients with hemorrhagic Bright's disease, nephrosis and essential hypertension. He reported no significant change in urea clearance, diuresis or blood pressure. Grant and Medes (42) have reported an elevation of the creatinine clearance in normal, unanesthetized dogs whose body temperature had been elevated by conventional diathermy. It is believed that in this animal creatinine clearance approximates the volume of glomerular filtration. Farr and Moen (43) have reported that fever induced in rheumatic patients by a heating cabinet was associated with a decrease of 25 to 38 per cent in the urea clearance. In two cases oliguria occurred even though large quantities of water had been ingested. Nicholes and Boynton (44) have found that an increase in the body temperature of dogs, produced by conventional diathermy or electromagnetic induction, was associated either with no change in creatinine clearance or a marked reduction. The evidence points to the idea that increased body temperature either has no effect on urea clearance or lowers the clearance, perhaps because of a circulatory adjustment to the hyperthermia. Chasis, Ranges, Goldring and Smith (45) have reported that pyrexia produced in human subjects by typhoid vaccine or pyrogenic inulin preparations very greatly increased the inulin clearance which is a measure of the volume of glomerular filtration. Renal blood flow was likewise much increased. Also, Nicholes and Boynton obtained a marked increase in the creatinine clearance by injecting typhoid vaccine into the same dogs that had shown no increase in clearance during the experiments of electrical heating. Furthermore, Smith (46) obtained the same effect of the pyrogen upon the inulin clearance and renal blood flow after the rise in blood

pressure, the chill and nausea and hyperthermia had been eliminated by pre-medication with amidopyrine. These studies strongly suggest that the increase in urea clearance which has been reported to occur in infections is not due to the hyperthermia but rather to the metabolic changes responsible for the fever.

*Miscellaneous.* The urea clearance has been reported to be within the normal range in psychotics even though over 20 per cent of the hospital admissions had albuminuria (47).

Page and Alving (48) reported that the operations of tonsillectomy, adenoidectomy and tooth extraction had usually no effect upon urea clearance. In 3 of 31 cases, however, the urea clearance was depressed in the days immediately post-operative. The first two operations were performed under gas-oxygen-ether anesthesia and the extractions under procaine anesthesia. MacKay (26) found a diurnal variation in the urea clearance of normal subjects and patients with Bright's disease. The standard clearance was depressed during the first hour after rising, after which it increased from 9 to 12 a.m. In this period there was the minimal variation. After lunch hour the clearance dropped and rose again in the late afternoon and evening. The standard clearance was much lower during sleep.

*Rate of filtration and degree of tubular absorption of urea.* In the normal kidney the magnitude of urea clearance is determined by the rate of glomerular filtration and the degree of tubular reabsorption. In the dog the effect of dietary protein and vitamin A upon urea clearance was by way of a change in the rate of glomerular filtration. Those factors which alter urea clearance over a considerable period of time probably do so by changing the glomerular filtration rate, rather than the relative amount of urea absorbed by the tubules. Normally, 40 to 50 per cent of the filtered urea is reabsorbed by the tubules and there are certain peculiarities about this absorption. Addis and Van Slyke early appreciated that urea excretion is less at low urine flows and Van Slyke came to the idea that in man urea excretion, at urine flows below 2 cc/min., varied as the square root of the urine flow, whereas above this flow it was independent of urine flow. Chesley (49) has reported that the formula of Van Slyke used in calculating standard urea clearance gave erroneously low results when the urine flow fell below a critical volume which he decided was about 0.35 cc/min. in adults. Below that urine volume the U/B reversed its trend and decreased with further decrease in urine volume. In a later paper he proposed that in oliguria "minimal" urea clearances be calculated by a formula provided with a constant.

Van Slyke, Rhoads, Hiller and Alving (7) found that in dogs with one kidney and it explanted subcutaneously about 6 to 12 per cent of the urea in the arterial blood was extracted by the kidney as the blood makes one renal circuit. However, they observed several times that the renal venous blood would have more urea than arterial, as much as 27 per cent more in some instances. Gordon, Alving, Kretzschmar and Alpert (50) found that extraction of urea by the explanted kidney of dogs was extremely varinble and that this was due to changes in the tubular absorption and not to changes in filtration. The absorption varied from none to all of the filtered urea and the average was between 40 and 50 per cent which average was usually approximated within any period of 30 to 60 minutes.

The urea clearance when determined on a rising urine flow is markedly higher than those values obtained with a corresponding urine flow but with the rate of flow constant or falling. This aspect has been discussed more completely elsewhere (2). It is not due to increased filtration since the urea clearance rises relative to the filtration rate. Recently Shannon (51) has studied urea excretion in the dog during forced diuresis. He reported that as the creatinine U/P ratio is reduced from 10, progressively less urea is reabsorbed. Inasmuch as he found that the urea/creatinine ratio could be extrapolated to 1.0 at a creatinine U/P ratio of 1.0 he considered this as evidence there is no active reabsorption of urea. He has explained the absorption of urea on the basis of the hypothesis that water reabsorption takes place at two sites in the nephron. The diffusion gradient created by the reabsorption of water in the proximal tubule accounted for the deficit in the urea clearance of the highest urine flow during water diuresis. The further deficit associated with low urine flows he attributed to diffusion in the distal portions of the nephron.

*Methods of determining the rate of glomerular filtration.* Convincing evidence for the idea that the inulin clearance measures the rate of glomerular filtration has been summarized by Smith (2). Richards, Bott and Westfall (52) have brought forth additional evidence that inulin is excreted solely through the glomerulus. Perfusion of inulin through the renal portal vessels of the frog's kidney did not result in the presence of inulin in the tubular lumen. In dogs and rabbits filtration in the kidney was temporarily abolished by lowering the renal blood pressure. The blood circulating through the kidney during this condition contained inulin and those substances, phenol red, diodrast and hippuran which have been shown to be secreted by the tubules. Although the urine subsequently obtained contained enough of the secre-

table substances to prove that secreting power of the tubules had not been abolished, it contained so little inulin as to warrant the conclusion that the tubules in dogs and rabbits are not capable of secreting inulin. Corcoran and Page (53) and Alving, Rubin and Miller (54) have developed methods of determining inulin colorimetrically, which method permits the analysis of samples containing very small amounts of inulin. Miller, Alving and Rubin (55) have reported the determination of inulin clearances in human subjects at very low plasma levels and found that these values closely approximated the clearances obtained at higher plasma concentrations. The results indicated to them that there was no tubular excretion or reabsorption of inulin.

Barnett (56) has developed an indirect method of estimating the inulin clearance and used it with infants and children. Inulin is injected intravenously and two samples of blood are drawn 2 and 3 hours later, the exact time after the injection being noted. On semi-logarithmic paper the inulin concentrations of the two blood samples are plotted on the logarithmic coördinate against time on the linear coördinate. The slope of a straight line connecting these two points is determined by the formula, slope $= \dfrac{K - \log C}{t}$, where $K$ is a constant whose value is determined by extending the line to zero time and taking the log of the concentration at this point; $C$ is the plasma inulin concentration in milligrams per cent at any given time, $t$, in minutes after the injection. The relation of the slopes of the lines obtained to inulin clearance as actually determined in children 5 to 15 years of age showed in Barnett's opinion a fairly good relationship between the two factors. He applied the method to 7 apparently normal full term newborns, 4 to 9 days of age, and found slopes corresponding to inulin clearances of 20 to 40 per cent of the normal, 120 cc. per minute. In 4 children, aged 14 days to $7\frac{1}{2}$ weeks, the slope of the lines corresponded to inulin clearances of 50 to 90 per cent. In 2 children 6 and 10 years, the slopes corresponded to normal clearances. In each group, the clearance was corrected for body surface area. This method may be very useful but needs further study. This report indicates that in the very young glomerular filtration is impaired which would not be expected on the basis of urea clearance determinations (22).

Rehberg (57), the first investigator who attempted to measure glomerular filtration in the human used the clearance of exogenous creatinine as a measure of this process. Later it was demonstrated by Shannon (58) that exogenous creatinine is excreted in the human by

glomeruli and tubules. Miller and Winkler (59) found that the clearance of endogenous creatinine in normal subjects was usually but not invariably equal to the inulin clearance. These workers also observed that in subjects with chronic nephritis and depressed renal function the endogenous creatinine clearance was usually higher than the inulin clearance. Another peculiar observation noted in this latter group of subjects was the failure of the exogenous creatinine clearance to rise regularly above the endogenous creatinine clearance after the injection of creatinine, as it does in the normal subject.

*Other clearance methods which may be used to measure certain aspects of renal function.* After the experimental demonstration by Elsom, Bott and associates (60) that diodrast and hippuran are excreted in large part by the renal tubules of the dog, rabbit and man, Smith and co-workers (61) have applied the diodrast clearance, because it is the maximum clearance known, as an indirect method of approximating the renal plasma flow. The theoretical principle of this method is that the kidney completely removes the diodrast from the plasma, so that if the concentration of diodrast in the arterial blood and the quantity of diodrast in a minute's excretion of urine are known, the calculation of renal plasma flow is simple. This method of determining renal plasma flow would be based on certain assumptions. One, that there was no storage of the diodrast in the tubular epithelium or any other cells of the kidney; second, that either the red cell membrane was completely impermeable to the diodrast or that there was free and rapid diffusion of diodrast across the red cell membrane, and third, that the plasma of the renal vein blood was free of diodrast.

Smith made certain experimental observations in normal human subjects which he interpreted as indicating a negligible store of diodrast within the kidney. Elevating the plasma concentration of diodrast depresses the ratio of phenol red clearance to inulin clearance and lowering the plasma diodrast permits increase in the ratio. The curves expressing the relationship between these two factors when obtained with rising and falling plasma concentrations of diodrast are not superimposable. However, when both curves are corrected for the delay time of excretion occasioned by the circuit substances must take from the site of injection to the collected urine sample, which time was experimentally determined by injecting phenol red intravenously and noting its initial appearance in the urine, the curves are superimposable except for a slight divergence at low plasma diodrast values. It would appear that the degree of sensitivity of the mechanism for excretion of phenol red to the presence

of diodrast is one uncertainty in Smith's interpretation. White (62) has reported that the diffusion equilibration rates of diodrast between cells and plasma in vivo were high enough in the dog so that the cell diodrast content of renal vein blood was lower than arterial cell diodrast. The blood cells therefore contribute diodrast to the urine. This behavior would give values for renal plasma flow that were too high. Furthermore, at arterial plasma concentrations of diodrast below that at which the clearance begins to be self-depressed, the extraction varied markedly and averaged 74 per cent. This failure of the kidney to free the plasma of diodrast completely in one passage of the blood would give values for renal plasma flow which would be too low. White, Findley and Edwards (63) found that the blood cells in the human contribute less diodrast to the urine than is the case with dog's blood. They also found that the diodrast plasma clearance begins to be self-depressed at lower plasma diodrast levels in the dog than in man which suggested to them that human kidneys extract diodrast more efficiently than do dog kidneys. These studies would indicate that in the dog renal plasma flow determined by the diodrast plasma clearance might be in error but in man the two errors are apparently smaller and since they are in opposite directions this method may closely approximate the renal plasma flow.

The physiology of the renal circulation has been quite recently reviewed by Smith (46). Certain features of this subject might be mentioned in this paper. Considerable experimental evidence indicates that the renal circulation possesses a great autonomy. The circulation through the kidney remains rather constant in spite of a changing arterial pressure. Also, this capacity of circulatory adjustment is not impaired by denervation. The renal circulation does not show the reactive hyperemia after a period of ischemia which is peculiar to other organs. It has been reported that the kidney vessels of the anesthetized dog do not participate in the reflex vasoconstriction produced by hemorrhage. It is also reported that the renal vessels in the dog are not directly affected by carotid sinus reflexes. Denervation does not produce hyperemia in the normal dog or man. After the renal artery is partly closed by a clamp, the renal blood flow returns spontaneously after some delay to the normal. The renal blood flow must be reduced about 40 per cent by the Goldblatt clamp in order to produce experimental hypertension in the dog. On the other hand, the oxygen arterial-venous difference approximates the normal (46). The experimental observations that anoxia in the frog results in constriction of

the glomerular arterioles may have some connection with this phenomenon. Adolph (64) directly observed that displacement of $O_2$ by nitrogen resulted in constriction of the glomerular vessels. At 22°C. only 4 per cent of an atmosphere of $O_2$ prevented this constriction and larger concentrations had no apparent effect on glomerular flow. Beck, Kempton and Richards (65) directly observed that when the perfusing fluid contained cyanide the glomerular arterioles constricted and other vessels in the kidney dilated, with the result that the renal blood flow was not severely curtailed. These experiments with the frog seem to suggest that in anoxia consequent to clamping of the renal artery, certain nephrons might be eliminated from the circulation and the others which receive blood extract $O_2$ at the usual rate.

The diodrast clearance in 9 normal women was found by Chesley and Chesley (66) to give an average renal plasma flow of 856 cc/min/1.73 $\bar{M}^2$. The range extended from 694 to 1233 cc. The plasma clearance of diodrast they found to be 18 per cent higher in the pregnant woman but they explained this by the hydremia of pregnancy. White, Findley and Edwards (63) obtained an average diodrast plasma clearance for 11 subjects of 497 cc/min/1.73 $\bar{M}^2$. Smith and associates (67) obtained a mean diodrast plasma clearance in 35 normal subjects of 669 cc/min/1.73 $\bar{M}^2$. The range extended from 455 to 921 cc.

Only 3 factors are known which produce a marked increase in renal blood flow. Unilateral nephrectomy in both dog and man result, in the course of a few months, in 70 to 100 per cent increase in the flow to the remaining kidney. In the unilateral nephrectomized dog a high meat diet increases renal blood flow to the explanted kidney. Typhoid vaccine or pyrogenic samples of inulin produce a marked increase in renal blood flow in man (46).

Simultaneous determination of renal plasma flow and the inulin clearance makes possible calculation of the filtration fraction or the extraction ratio of inulin, by which term is meant the per cent of the plasma inulin which is filtered at the glomerulus. Inulin clearance/renal plasma flow $\times$ 100 = filtration fraction. An increase or decrease in the filtration fraction is generally interpreted as an increase or decrease respectively, in the tone of the efferent arteriole. The renal hyperemia produced by a pyrogen is considered to be brought about by dilatation of the efferent arteriole inasmuch as the filtration fraction markedly falls. Adrenalin, orthostatic vasoconstriction and psychogenic vasoconstriction, all increase the tone of the efferent arteriole (46).

Inasmuch as the filtration rate can be determined by the inulin clear-

ance, certain functions of the renal tubules can be quantitatively measured. These possibilities have been pointed out by Smith (68). Shannon and Fisher (69) have demonstrated that when blood glucose is elevated sufficiently, the rate of reabsorption of glucose by the tubules in the dog becomes maximal and rather constant. This has been shown to hold for the human kidney and the maximal rate of glucose absorption can be determined and is designated glucose Tm. Glucose Tm would evaluate the number of normal active nephrons and the tubular absorptive mass of the kidney since the maximal quantity of glucose reabsorbed would require the nephron to have a functional glomerulus and tubule with this absorptive capacity. Elevation of plasma diodrast sufficient to exceed the excretory capacity of the tubules would give diodrast Tm or the maximal quantity of diodrast excreted by the tubules per minute. This value would serve to measure the mass of active excretory tubules, whether glomerular or aglomerular. Inulin clearance of course measures all functional glomeruli regardless of whether the tubule is physiologically functional or impotent. Goldring, Chasis, Ranges and Smith (67) found an average glucose Tm of 344 mgm./min. corrected to a surface area of $1.73/\bar{M}^2$ in 14 subjects with no evidence of renal disease. They have pointed out certain factors which might influence the accuracy of this determination. The rate of glomerular filtration and blood glucose must be very accurately determined since a small error greatly changes the amount of glucose filtered. The amount of glucose delivered to the tubules should exceed the maximal rate of glucose absorption by at least 20 per cent. Furthermore, the elevation of plasma glucose to the necessary 350 to 700 mgm. per cent probably causes expansion of plasma volume which might disturb the normal equilibrium in the kidney. This same group found the diodrast Tm in 35 subjects ranged from 36.6 to 72.0 with a mean of 51.6 mgm. of iodine per $1.73/\bar{M}^2$ per minute. White and associates (63) obtained a value of 44 mgm.I./min./1.73 $\bar{M}^2$ for diodrast Tm on 3 normal subjects.

*Clearances in diseases of the kidneys.* The changes in the urea clearance of 67 patients with Bright's disease, the hemorrhagic, sclerotic and degenerative types, have been reported by Van Slyke and associates (70) and the effect of renal disease upon urea clearance generally discussed by Peters and Van Slyke (71).

Hayman and Johnston (72) have compared the urea clearance with estimates of the number of glomeruli in one kidney after postmortem injection. They considered that in cases of arteriosclerotic Bright's

disease and probably in chronic hemorrhagic Bright's disease, reduction in urea clearance was associated with a decrease in the number of injected glomeruli. In pneumonia and pyelonephritis, urea clearance was greatly reduced in the presence of a normal number of glomeruli. Hayman, Martin and Miller (73) have recently reported similar studies on this same problem. Seventy-nine patients were studied. Nineteen showed no evidence of renal disease during life and none or slight was found at autopsy. These served as controls. There were 2 patients with sub-acute and 7 with chronic glomerulonephritis. Thirty-nine patients had arteriolar nephrosclerosis of varying degree. Twelve patients had low renal function during life but on postmortem examination only slight changes were found and the number of glomeruli was normal. They found poor correlation between urea clearance and renal weight. However, the mean urea clearance fell with a decreasing number of glomeruli but not directly, since the clearance was reduced more rapidly then the number of nephrons. In contrast to this is their observation, upon dogs, whose renal mass had been reduced by sub-total nephrectomy, that the percentage reduction in clearance was less than the reduction in the number of glomeruli. In certain cases of acute infections and jaundice, the clearance they found might be markedly reduced in spite of a normal number of glomeruli showing no significant histologic changes.

Cullen, Nelson and Holmes (24) have reported urea clearances of 15 children during the acute stage of and convalescence from hematuric nephritis. The values were reduced but in a majority of the cases the clearance had returned to normal within a month after cessation of symptoms. In a group of 78 children with a history of acute hematuric nephritis, the distribution and means of the urea clearance values coincided with those cases having no history of renal disease. Apparently acute nephritis leaves little or no residual renal damage in children.

Chasis and Smith (74) have determined inulin and urea clearances in 7 patients during a first attack of diffuse glomerulo-nephritis and in 15 patients with chronic nephritis. In 3 cases during the acute diffuse nephritis, the inulin clearance was markedly reduced. In 13 of the cases of chronic nephritis the inulin clearance was greatly reduced. The changes in urea clearance paralleled those in glomerular filtration and there was no evidence of increased tubular reabsorption of urea. As a matter of fact, tubular absorption was impaired because the U/P ratio in the cases of nephritis fell as low as 2, whereas in the normal it has never been observed by these workers to fall below 6 and frequently

could not be reduced below 10. This indicated impairment in the capacity to absorb water and as the U/P ratio fell from 10 to 2, the reabsorption of urea decreased and the urea/inulin clearance ratio rose toward 1.0. Arkin and Popper (75) have reported that there is less tubular absorption of urea in cases of acute glomerulonephritis, benign hypertension and nephrosis. However, they used endogenous creatinine clearance to measure glomerular filtration and this has been found to exceed the inulin clearance in kidney disease. Miller, Alving and Rubin (55) have determined inulin clearances in patients with nephrosis, chronic and subacute glomerulo-nephritis and essential hypertension. They were made at plasma levels of inulin from less than 5 to 96 mgm. per cent. Since the clearances did not seem to be affected by the level of plasma inulin, there probably was no tubular excretion or absorption of inulin. In the cases of chronic glomerulo-nephritis the inulin clearance was moderately to greatly reduced. There was less reduction in the cases of nephrosis and perhaps none in those of hypertension. Chesley and Chesley (76) have studied renal blood flow, by means of the diodrast clearance, in 37 women with hypertension and renal impairment occurring separately and together. Hypertension sometimes occurred with the total renal blood flow within normal limits. In other cases, the deficit in renal blood flow was only slight. However, in the majority of patients with essential hypertension the diodrast clearance was considerably reduced below the average normal. Such deficit in total renal blood flow seemed often to be associated with efferent glomerular arteriolar constriction and consequent, high filtration fraction which might maintain, in their opinion, normal renal function as determined by other tests. In the hypertension seen in women who had toxemia of pregnancy as the initial phase of the hypertension, the filtration fraction was usually normal. They suggested that the glomerular capillaries rather than the efferent arterioles were the site of resistance to blood flow in these cases. The urea clearance often paralleled the renal blood flow. The exception appeared in those cases where efferent glomerular arteriolar constriction existed. In 70 subjects the coefficient of correlation between the urea clearance and blood flow was 0.79. These workers decided that in most cases the renal blood flow could be predicted within 15 per cent from the urea clearance.

In a series of hypertensive subjects Smith, Goldring, Chasis and Ranges (77) found invariably that the renal blood flow was less than normal values and the filtration fraction greater than was observed either in the normal subject or in subjects with chronic glomerulo-

nephritis of the same degree of renal impairment. Hypertensive subjects in whom the renal mass was not perceptibly reduced, although showing absolute renal ischemia, had a filtration rate and urea clearance which were normal, probably in part because of the increased tone of the efferent glomerular arteries and in part because of increased systemic arterial pressure. In hypertensive subjects in whom the excretory renal mass was markedly reduced the renal blood flow and filtration rate, although reduced absolutely, might be relatively increased when referred to the mass of the residual normal tissue.

Corcoran and Page (78) found that the slow intravenous infusion of preparations containing renin or angiotonin into conscious, trained dogs with only one kidney and it explanted subcutaneously or with both kidneys located subcutaneously resulted in increased arterial pressure, reduced renal blood flow and an increase in the extraction or filtration fraction of inulin from the blood. These results indicated that the efferent arterioles of the nephrons had increased tonus. These substances apparently produce in the dog's kidney a condition quite similar to that observed in clinical hypertension.

Concentration and dilution tests. *Relationship of urine volume to glomerular filtration.* The specific gravity of urine is an expression of the concentration of its dissolved materials. This concentration is determined by the volume of water permitted to reach the urinary bladder, the total amount of urinary solids produced by bodily metabolism and the relative amounts of these solids which escape absorption by the renal tubules. The urine volume is determined normally by the relative amount of water reabsorbed by the tubules and not by changes in the filtration rate (2). Under physiological conditions the filtration rate and urine flow can be varied over a wide range of variation with no evidence that the two factors are dependent upon each other (13, 19, 79). The important rôle of tubular absorption of water in regulating urine volume is seen in a comparison of the glomerular filtration rate in man of about 120 cc. per minute to the urine volume of about 1500 cc. per 24 hours. Furthermore the xanthine and mercurial diuretics apparently increase urine flow independently of any effect upon the filtration rate (2).

*Tubular reabsorption of water.* Water reabsorption may occur throughout the length of the tubule. Richards found that in the frog the chief site of water absorption was in the proximal part of the distal tubule. He also obtained evidence indicating that water absorption occurred to some extent in the proximal tubule. In some experiments with phlori-

zinized frogs, it appeared that 20 per cent of the water filtered underwent absorption in the proximal tubule (80). In a survey of the comparative physiology of the kidney, Marshall has brought forth the evidence that the thin segment of the loop of Henle plays an important rôle in water absorption. It is only in those animals possessing this structure or some semblance of it that a hypertonic urine is produced. This is restricted to mammals and some of the birds (81). Furthermore Burgess, Harvey and Marshall (82) found that the anti-diuretic factor exerted its effect only in animals possessing the thin segment of the loop of Henle.

The polyuria of diabetes insipidus indicates a failure of part of the physiological mechanism influencing the tubular absorption of water. Fisher, Ingram and Ransom (83) in a very comprehensive review of this problem express their concept of its pathological physiology. "The supraoptico-hypophyseal system regulates the secretion of the antidiuretic hormone by the neural division of the hypophysis . . . the neural division includes the infundibular stem and median eminence. The interruption of the supraoptico-hypophyseal tracts in the hypothalamus causes the neural division to become atrophic and functionally inactive and leads to a deficiency of the antidiuretic hormone in the organism. Likewise, section of the stem high enough to cut all of it and the median eminence away from the hypothalamus brings about a similar atrophy and deficiency. Extirpation of the neural division in all its parts leads to the same hormonal deficiency by virtue of the fact that it removes the site of formation of the antidiuretic principle." Inasmuch as hypophysectomy does not produce polyuria, the above workers consider that the anterior lobe controls the diuretic processes in the body not through a specific diuretic hormone but rather through its general control over metabolism and activity. These workers speak of diabetes insipidus as being caused by a disturbance of the equilibrium normally obtaining between the neural division and the pars anterior.

Verney's experimental studies have indicated to him that the hypophysis is the chief regulating influence in the maintenance of water balance. The inhibitory effect of emotion, muscular activity, pain and fear upon urine flow he considered as due to the pituitary mechanism. The renal nerves and adrenalin were shown not to be a factor in his experiments(2).

On the other hand, the results obtained by Fee and Newton and Smirk (2) indicated that the pituitary was not essential for the control of water diuresis. In decerebrate, hypophysectomized dogs and cats the course of water diuresis was very similar to the control animals

possessing the pituitary mechanism. Furthermore, they showed that when diuresis was obtained more than once after hypophysectomy, the second or third diuresis showed no decrease either in the delay of onset of diuresis or in the delay in attaining the maximum urine flow. They concluded that the delay in onset of diuresis after water administration is independent of the anti-diuretic factor of the pituitary, which conclusion is opposed to Verney's concept that the delay in onset of diuresis was due to the removal or nullification of the anti-diuretic hormone.

Furthermore, the concept that the variable concentration of the anti-diuretic hormone in the blood regulates the degree of water absorption by the tubules under normal physiological conditions fails to explain one important fact. Under the conditions of either extreme water diuresis or diabetes insipidus the urine volume hardly exceeds 20 per cent of the volume of glomerular filtration. Smith (2) has designated the 80 per cent which is always normally absorbed as the obligatory reabsorption. This may be due to the increased colloidal osmotic pressure of the plasma which results from the glomerular filtration and the lowered hydrostatic pressure in the peritubular capillaries. The remaining 20 per cent, the facultative reabsorption, Smith considers to be under the influence of the anti-diuretic hormone. It is the removal of this fraction which results in a hypertonic urine.

*Effect of the urinary constituents upon specific gravity.* The relative influence of the different urinary constituents on the specific gravities obtained in the concentration tests has been considered by Alving and Van Slyke (84). On the basis of data given in Landolt-Börnstein's "Tabellen" of the effect of those various inorganic salts, which are found in the greatest amounts in urine, upon the specific gravity of water, they calculated that in a urine of specific gravity 1.030 about $\frac{2}{3}$ of the rise above that of water might be attributed to the mineral salts and about $\frac{1}{3}$ to the urea. The ions $SO_4$, $HPO_4$ and $H_2PO_4$ would be expected to have a much greater effect in elevating specific gravity than similar concentrations of other substances found in urine. An experimental study of this problem has been reported recently by Price, Miller and Hayman (85). These workers established the generalization that the specific gravity of a urine could be calculated if the chemical analysis of its major constituents were known. They prepared artificial urines, approximating natural urine in composition and, using data from Landolt-Börnstein or International Critical tables, calculated the specific gravity of such urines. The maximum difference between calculated and observed specific gravity in 5 cases was 0.0007 and averaged 0.0004.

Also after a weighed amount of solid was dissolved in urine, the average difference between observed and calculated specific gravity was 0.005. Furthermore, when a concentrated urine was diluted with water, specific gravity increment was always a linear function of the degree of dilution.

The above workers determined the composition of the urine of human subjects receiving diets of 40, 50, 100 and 110 grams of protein. In urine from subjects on a low protein diet, urea accounted for 15 to 20 per cent, chlorides for 25 to 30 per cent, sulfate and phosphate together 15 to 25 per cent, bicarbonate 1 to 5 per cent and creatinine 1 to 2 per cent of the observed specific gravity. On a high protein diet, urea, sulfate and phosphate contributed a slightly higher proportion to the specific gravity. The undetermined fraction of specific gravity comprised 10 to 30 per cent and in the same urine the excretion of undetermined solids amounted to 10 to 15 grams daily or from 10 to 30 per cent of the total solids. The undetermined solids are composed of organic substances, are low in nitrogen, are dialyzable and are largely of endogenous origin. The various coefficients proposed for estimation of total solids in urine from specific gravity were found by Price, Miller and Hayman to be valid only for urines of the same relative composition.

The studies of Gamble, McKhann, Butler and Tuthill (86) upon the relation of urine volume to the amount of various urinary substances excreted by rats given free choice of water is pertinent. The water requirement established for the individual substances such as Na, K, Cl, $HCO_3$, $H_2PO_4$ and $SO_4$ remained additive when mixtures of them entered the urine. In the presence of urea, water expenditure was found to be much less than the sum of the requirements for urea and the accompanying substances as separately determined. This economy was not demonstrated with urinary glucose or galactose. The kidney may not be the only factor in these experiments making for water economy in the excretion of urea inasmuch as water consumption was not controlled. However, this behavior of urea would tend to elevate the specific gravity of the urine.

*Factors affecting the excretion of electrolytes.* The renal excretion of water and inorganic salts has been reviewed by Peters (87) especially in relation to the homeostasis of body fluids. The urinary excretion of sodium, potassium, calcium, chloride, phosphate and sulfate was discussed by Smith (2) in 1937, and later brought up to 1939. Most of the electrolytes in the plasma act like "threshold" substances, in that they are largely retained when their plasma concentration is low and are rapidly excreted when the plasma concentration is elevated. The uri-

nary excretion of chloride is influenced by the level of plasma chloride. In man the excretory rate is about 5 mgm. of NaCl per hour when the plasma Cl is below 95 mM per liter. Above this level the Cl excretion increases rapidly. Water diuresis increases the loss of both Na and K. Diuresis caused by NaCl increases the loss of K and KCl similarly affects the excretion of Na. For potassium, apparently there is a renal threshold of about 3.5 mM per liter and above this plasma concentration, K is rapidly excreted (88). Hall and Langley (89) found that the potassium clearance in 3 normal subjects was constant over a wide range of urine flow but below a flow of 0.6 cc/min./$\overline{M}^2$ the clearance fell sharply.

Adrenal insufficiency affects the urinary excretion of Na and K. Loeb and associates (90) conducted balance studies before and after adrenalectomy in 3 dogs. An increase in blood potassium was not obviously correlated with changes in the K balance. The balance of Na showed a striking loss of Na during development of adrenal insufficiency. After adrenalectomy, the urine volume, and both the concentration and total amount of urinary Na were increased. The behavior of Cl paralleled that of Na but its loss was not equivalent to that of Na. Cessation of the injections of cortical extracts in adrenalectomized dogs is associated with increased excretion of Na which reaches its maximum in 48 to 72 hours. The concentration of Na may be nearly doubled in this period even though the urine volume is greatly increased. After 3 days, the urinary excretion decreases because, according to Harrop (91), of depletion of the body's reserves of Na. Anderson, Joseph and Herring (92) reported that 24 hours after adrenalectomy in rats there is an increased urinary excretion of radio-active Na and retention of radio-active K. Also, adrenalectomized rats receiving 10 per cent NaCl, at first excrete administered radio-active Na like normal control rats, but later show Na retention. The excretion of radio-active K was the same as the controls (93). Harrison and Darrow (94) determined the volume of glomerular filtrate and simultaneously the urine/plasma ratios of Na, K and P in the adrenalectomized dog under various conditions. They concluded that following adrenalectomy the renal tubules fail to reabsorb Na adequately from the glomerular filtrate at a time when the concentration of Na in the plasma is low and fail to excrete K and phosphate when these ions are abnormally concentrated in blood plasma. The excretion of these ions was expressed in terms of the ratio of their concentration in urine (U) to that in plasma (P). However, the U/P ratio does not indicate the magnitude of the tubular reabsorption of these ions because it does not consider the rate of glomerular filtration

and the per cent of the filtered water which is reabsorbed. Using their data as given, this writer has calculated the clearances of creatinine, Na and K and the per cent of the filtered ions that is absorbed by the tubules. These calculations showed that in adrenal insufficiency the percentage of the filtered K which was reabsorbed was either about the same as normal or much less than normal. Administration of Na or Na plus the cortical extract greatly reduced the percentage reabsorption. This effect together with the increased glomerular filtration would explain how therapy corrects the plasma accumulation of K. Adrenal insufficiency did not materially change the tubular reabsorption of Na, inasmuch as with one exception 99 per cent or more of the filtered Na was reabsorbed. This result is not in agreement with the finding that the urinary Na is increased in adrenal insufficiency. The reason for the discrepancy is not apparent. The administration of Na in adrenal insufficiency reduced the percentage reabsorption of Na. In this respect the tubules of the adrenalectomized dog respond as in the normal dog.

Mainzer (95) found that in patients remaining in bed with a normal intake of food, the ratio of Na:Cl varies greatly in the urines collected in a 12 hour day as well as 24 hour periods. Tests carried out over a long period revealed that the excretion of Na and Cl occurred in equivalent quantities. Clinical use of this ratio according to Mainzer must be limited to prolonged observation periods.

Since Smith's (2) discussion of the excretion of sulfate two papers have appeared on the subject. Goudsmit, Power and Bollman (96) found in the dog that the clearance of the naturally occurring concentrations of sulfate in the plasma approximated 8 per cent of the creatinine clearance, indicating that about 92 per cent of the filtered sulfate is absorbed by the tubules. Increasing the rate of urine flow decreased the fraction which was reabsorbed. After the intravenous injection of sulfate its clearance rose markedly and at concentrations of 180 to 200 mgm. per cent it was about 90 per cent of that of creatinine. All or nearly all of the sulfate in the serum of dogs could be considered filterable through collodion membranes. Bjering and Øllgaard (97) found the endogenous sulfate clearance in normal persons to average 37. They reported that it increased with the administration of sulfate, approaching a constant value between urea and creatinine clearances. It was generally lower in patients with kidney disease, corresponding with the fall in creatinine and urea clearances. They also reported that the straight line curve obtained by plotting the concentration of blood sulfate against the sulfate clearance could not be extended through zero

concentration of blood sulfate but crossed the concentration at 0.8 to 0.9 mgm. per cent. This type of curve they interpreted as indicating that this much of the blood sulfate was not filterable at the glomerulus. They state that after correcting for this non-filterable fraction the sulfate clearance has the same numerical value as those reported for inulin clearance. Such a statement without the simultaneous determination of inulin and sulfate clearances is hardly acceptable. Furthermore, Hayman (98) found no evidence from similar curves that part of the blood sulfate in the human was not filterable at the glomerulus.

The specific gravity of urine is therefore the resultant of a number of physiological processes carried on within the kidney. To these renal factors should be added the extra-renal factors pointed out by Barker (99) such as impairment of the systemic circulation and the hydropigenous states of salt retention, dehydration and inanition. Lewis and Alving (25) found that the maximum specific gravity declined with those ages above 40 years. At 40, the specific gravity was 1.030 and at 90 years it was 1.023. Manchester (100) has reported a diurnal rhythm in water and mineral excretion. The urine volume and the urinary Na, K and Cl were greater during the day than at night. Only slight fluctuations were noted in urinary phosphate, sulfate, titrable acidity and ammonia. The water balance was found to be negative during the day and positive at night. The rise in specific gravity of night urine he decided was largely due to increased concentration of the nitrogen compounds. In dehydration the diurnal rhythm in Na excretion was temporarily reversed.

*Specific gravity of urine in nephritis.* In nephritis the specific gravity of the urine undergoes changes which have been considered of great importance in evaluating renal function in this condition. The specific gravity of urine in acute nephritis varies, apparently depending upon urine volume. With oliguria the specific gravity is usually 1.020 higher. The histological picture usually shows much ischemia of the glomeruli and swollen endothelia of the glomerular vessels (101). This of course greatly lowers filtration pressure in the glomeruli so that the rate of glomerular filtration is much reduced. Reference has already been made to the experimental finding of reduced glomerular filtration in acute nephritis. Presumably the slower rate of filtration would allow more time for the tubular reabsorption of water leaving a more concentrated urine. In chronic nephritis there is polyuria and the specific gravity of the urine is not only reduced but does not show the variations found under normal conditions. The possible mechanism of these uri-

nary changes interested Bradford (102) because although many nephrons are rendered functionless by disease, those remaining should excrete a normal urine. He found that surgical excision of portions of both kidneys in dogs resulted in a great increase in urine volume. If one-third of the total kidney weight is left, the animal remains in good health, the urine volume is increased and the animal cannot concentrate the urine. Using a heart-lung and two kidney preparations, Verney (103) reported that ligature of the anterior branch of the renal artery of one kidney resulted in a decreased renal blood flow and no diminution in urine flow. After a short time the urine flow gradually increase above that of the control kidney. In some cases the urine flow was thirty times the control. The chloride concentration of the urine usually did not change but the minute output increased. The concentration and minute output of urea immediately fell but was greater than that expected on the basis of the amount of kidney still being perfused. A similar result was obtained by Verney in the unanesthetized dog by ligature of one renal artery. The urine flow from the normal kidney within an hour increased over its control rate and within 3 hours was nearly three times as great as that of both kidneys during the control observation. Injection of pituitary extract did not diminish the polyuria.

Rytand (104) statistically studied the data obtained in a number of nephritic patients. He reported a curvilinear relationship between specific gravity of the urine and the Addis ratio. The kidney was able to excrete a concentrated urine until the ratio had decreased to 80 per cent of the normal. Below this value the urine's specific gravity rapidly decreased until the ratio was 30 per cent of normal.

Hayman, Shumway, Dumke and Miller (105) have recently reviewed the literature of, and reported their own experimental study of hyposthenuria, or the inability to concentrate the urine. They found that hyposthenuria in dogs could be produced in a number of ways. These included reduction in kidney mass, uranium poisoning, ureteral obstruction, renal denervation and low protein diets. The reduction in functioning kidney mass was accomplished by surgical removal of about one-third of one kidney and later complete removal of the remaining kidney. The concentration tests, after complete recovery, showed a urine of increased volume and decreased specific gravity. It was found that these dogs could excrete a concentrated urine, under certain conditions such as increased concentration of plasma colloids, low blood pressure and intravenous injections of sodium sulfate. Adrenal cortical

hormone and pituitrin were without effect in causing such kidneys to concentrate the urine. These workers thought that the polyuria and hyposthenuria in these dogs were due to increased blood flow to the remaining nephrons with a resultant increased volume of glomerular filtrate per nephron. The return to a normal concentrating capacity effected by a reduction in blood pressure and the curvilinear relationship found between creatinine clearance and the per cent of glomeruli remaining post-operatively in these dogs are in agreement with this idea but unfortunately these workers did not determine the renal blood flow or calculate the per cent of the filtered water which underwent tubular absorption in the normal and post-operative conditions.

In uranium poisoning, presumably with a predominantly tubular lesion, the inulin, creatinine and urea clearances were reduced and there was an increased volume of dilute urine. The lowered clearances were attributed by these workers to failure of the damaged tubules to prevent diffusion of these substances into the peritubular capillaries. A urine of high specific gravity was not obtained from dogs having renal tubular damage, probably because of the back diffusion.

Injection of pituitrin enabled the denervated kidney to excrete a concentrated urine. The hyposthenuria in dogs with a low protein diet was probably due to the small amount of urinary solids produced by bodily metabolism.

Reference has been made to the study of renal function and the number of glomeruli in the human kidney by Hayman and associates (73). They found upon plotting the maximum specific gravity of the urine against the number of glomeruli which was determined post-mortem in cases of chronic glomerulonephritis and nephrosclerosis, that the specific gravity fell with the number of glomeruli until the latter reached about 750,000 glomeruli per kidney, after which it remained fixed at about 1.010 in spite of further reduction in the number of glomeruli.

A hepatorenal syndrome has been discussed in papers from clinicians. Nonnenbruch (106) has written of a nitrogen hyposthenuria as occurring in cases of liver disease such as catarrhal icterus, vascular congestion, lues, cirrhosis and carcinoma of biliary tract. According to Nonnenbruch, there may be oliguria, hyposthenuria and renal insufficiency or polyuria, hyposthenuria and no insufficiency. Boyce and McFetridge (107) have also written of a hepatorenal syndrome and express the idea that obstruction to the biliary tract damages the liver which then causes oliguria, anuria and death. Both of these clinical papers are characterized by vague, worthless generalities and extreme paucity of scientific

data. A direct, specific effect of the liver upon renal function remains to be demonstrated.

The polyuria, hyposthenuria and relative fixation of the urine's specific gravity, when part of the kidney substance is removed, is a striking physiological reaction. Verney (103) refers to the normal condition as one in which a part of the organ inhibits the activity of the other part. He considers that in this response the physiological reserve of the remaining nephrons has been drawn upon. It would appear that the reaction serves a compensatory function but the actual mechanism remains to be elucidated. This reaction of the kidney to a loss of kidney substance may explain the polyuria and the reduction of the specific gravity of urine in chronic nephritis.

As the nephritic process advances on the kidney, the maximal concentrating capacity of the kidney is reduced. On the basis of the preceding discussion, a number of factors might be involved. Since the range of specific gravity of the urine is between isotonicity and hypertonicity, the water absorbing capacity of the thin segment of the loop of Henle might be greatly impaired. In support of this idea is the reported failure of pituitrin to prevent the polyuria of chronic nephritis. There might be a failure on the part of the tubule to prevent the diffusion of the urinary solids into the peritubular capillaries. Hayman and coworkers (105) considered that this occurred in the experimental nephritis produced by uranium. It has been reported that sulphate retention in the blood occurred before that of urea. This might be due to increased absorption of the sulfate but it awaits experimental study. Impairment in the capacity of the kidney to dilute the urine probably indicates reduction in the ability of the tubules to reabsorb the electrolytes. Peters, Wakeman and Lee (108) found that in advanced nephritis both base and chloride were excreted in the urine when serum Cl had fallen below the level which in a normal individual determines achloruria. Arkin and Popper (75), using the endogenous creatinine clearance as a measure of glomerular filtration, reported that there is less tubular absorption of urea in patients with damaged kidneys. When the maximum specific gravity reaches 1.010 the renal tubules have practically ceased to exercise any selective rôle in the reabsorption. Since this value approximates that of an ultra-filtrate of blood plasma, namely 1.007, if the tubules are reabsorbing any of the glomerular filtrate, it is solely by physical diffusion, and the composition of the absorbate would be about the same as the glomerular filtrate. Failure of the nephritic kidney to lower the specific gravity below 1.010 in the dilution test indicates

that the tubules cannot selectively reabsorb salts. In other words when the specific gravity of the urine becomes relatively fixed at 1.010 the tubules have become merely inert tubes through which a fluid resembling an ultra-filtrate of blood plasma may pass either to the collecting ducts or to the peritubular capillaries.

*Comparison of urea clearance with some of the other tests of renal function.* Van Slyke and associates (109) have reported that in patients with diminishing renal function, the urea clearance shows evidence of the diminution sooner than does the blood content of creatinine or urea or the phenolsulfonephthalein. Alving and Van Slyke (84) have compared the results of concentration and dilution tests with the urea clearance in nephritic patients. They concluded that the concentration tests, done with proper care, were sensitive for qualitative detection of damaged renal function. For measuring the extent of renal damage, however, the concentration tests did not appear to be suitable. Although the extent of fall in urine concentration showed statistical correlation to the urea clearance, the disagreement in given cases might be extreme. A patient may have practically recovered from nephritis, with a normal urea clearance and yet excrete urine of specific gravity 1.009 to 1.012 which is the same as that of a patient in terminal uremia with 3 to 5 per cent of normal clearance. Freyberg (110) has discussed the choice of tests of renal function. He compared in patients with hemorrhagic nephritis, urea clearance, the P.S.P. for 15 minutes and the concentration tests. He decided that generally a parallelism existed between the three tests. In the initial stage of nephritis concentrating power was lost and during recovery the urea clearance became normal while concentration was still impaired. In arteriosclerotic Bright's disease there was more difference between clearance and concentration. In 4 cases, clearance was lowered with normal concentration. In 38 cases there was normal clearance and impaired concentration. In 4 cases the clearance was elevated and concentration impaired. He concluded that the concentration test was more sensitive in this series of cases in whom the renal damage was less severe. In 12 of these cases there was a low dye excretion with normal concentration. Perhaps one factor which operates to make urea clearances appear less sensitive than the concentration test is the great variation of urea clearance in normal subjects. Although 75 to 125 per cent is generally considered normal, a subject may have lost 50 per cent of his clearance and yet be considered in the normal range. This individual variation does not appear in carefully done concentration tests. Clearances seem to have the greater

value when it is desired to measure renal function at intervals over a considerable period of time.

This survey of the factors influencing renal function did not yield much information about the physiological regulation of renal activity. Although many factors may influence the various aspects of renal activity the fundamental control is yet to be learned. Even the hypothalmico-hypophyseal mechanism apparently is chiefly concerned with raising urine from isotonicity to hypertonicity or with the tubular absorption of about 20 per cent of the filtered water. The excretion of the electrolytes and of glucose has a certain kind of regulation by the threshold mechanism, that is, when the amount filtered per minute exceeds the amount reabsorbed by the tubules, the excess is excreted. It might be that renal function proceeds at the maximum capacity. However, Richards and Schmidt (111) directly observed intermittence in glomerular activity of the frog. Khanolkar (112) injected carmine or hemoglobin intravenously into rabbits and upon microscopic examination found more of the injected substance in some glomeruli than others. Hayman and Starr (113) intravitally stained the glomeruli of rabbits by injecting Janus green B into the aorta. Wide variations in the number of glomeruli open to the dye occurred spontaneously. These workers reported that caffein and salt solution increased the number of open glomeruli, whereas adrenalin and $CO_2$ inhalations usually reduced the number of open glomeruli. On the other hand, White (114) has reported observations on glomerular perfusion made on 17 kidneys of 11 dogs and 13 kidneys of 7 rabbits following injection of a carbon suspension into the renal artery. He found that either all the glomeruli were injected or that the distribution of the uninjected glomeruli was such as to favor the idea that failure of injection was due to the distribution of ink in the larger pre-glomerular arteries rather than to closure of glomeruli. The question of intermittent, sub-maximal or maximal activity of the nephrons remains confused. From the physiological viewpoint this is an important problem because if renal activity proceeds at a maximal rate, a search for factors regulating this activity would likely prove fruitless. In applying the tests of renal function to the diseased kidney, one might consider that part of any impairment of function might be due to renal structural changes. Hayman (115) upon perfusing kidneys removed at autopsy, found that those kidneys of benign arteriolar sclerosis, acute and chronic diffuse glomerulonephritis, yielded less perfusate at various perfusion pressures than did normal kidneys. The kidneys showing only degenerative changes gave the

same volume of perfusate as the normal. In addition to these structural changes, there may be purely functional changes which might be responsible for the renal impairment. Some of the more recent clearance methods may make possible a differentiation between these two types of causes for renal impairment.

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# ANAPHYLAXIS

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An anaphylactic reaction may be defined as a reaction that is produced in an animal by the administration of a foreign substance, the reaction occurring in an animal sensitized to that substance by a preceding suitable exposure, the nature and severity of the reaction depending upon the kind and degree of the sensitization of the animal and not upon any toxic properties of the foreign substance, so that the reaction may be said to be characteristic for the animal rather than characteristic for the foreign substance. The anaphylactic reaction is one of the expressions of an antigen-antibody reaction. Correspondingly there are numerous problems concerning the nature of the antigens and antibodies involved, methods of sensitization and of inducing the reaction, the phenomena of desensitization and refractory state, passive transfer of the sensitized state, etc. These may be considered as immunological problems and they will not be dealt with in this discussion except as they impinge upon the argument. The explosive and violent character of the anaphylactic reaction, with marked effects manifested in the respiratory, circulatory, glandular and smooth muscle systems, presents the problem of the proximal pathogenesis of these symptoms that is no less intriguing than the more fundamental immunological problem. Even with this narrowing of scope the literature is too extensive to permit more than an appraisal of the present status and the work that has seemed to be pertinent thereto.

Concurrently with the accumulation of a wide variety of experimental observations regarding the conditions under which an anaphylactic reaction might be obtained, numerous theories to explain the manifestations were elaborated (14, 33, 39, 55, 56, 58, 67, 71, 73, 79, 88, 89, 90, 91, 102, 130, 138, 149). Space does not permit a detailed consideration of either these theories or the varieties of evidence submitted in their support. Suffice it to say that a mass of evidence has accumulated to establish that the anaphylactic reaction is indeed the product or biproduct of an antigen-antibody reaction. Furthermore there is sub-

stantial evidence that it is the antibodies attached to fixed tissue cells which are most concerned in this reaction. This evidence is most abundant and convincing in the cases of the dog and the guinea pig. As will be referred to later, it may not be so true for the rabbit and perhaps other animals. Manwaring (82), Pearce and Eisenbrey (98), Coca (21), and Fenyvessy and Freund (52) have shown that the blood of a sensitized animal can be removed and replaced with the blood of a normal animal without impairing the sensitivity of the animal. Various workers (see 75 for references) have reported that there is a necessary latent period for the passive sensitization of normal animals by the injection of antiserum, which fact is difficult to explain if antigen and antibody react in the circulating blood, but fits in well with the assumption that a latent period is necessary for the antibody to enter or become attached to the fixed tissue cells and thereby render them sensitive. Schultz (115) and Dale (27) demonstrated that isolated tissues (intestine, uterus) of sensitized animals, washed as free as possible from blood, show a specific or anaphylactic contraction when antigen is added to the bath in which they are suspended. Finally, evidence has been presented by Weil (139) and by Dale and Kellaway (29, 30) indicating that an abundance of free antibody instead of favoring the occurrence of a reaction actually interferes with the anaphylactic reaction presumably by binding the antigen before it can reach the sessile receptors within the fixed tissue cells.

Despite the apparently incontrovertible mass of evidence for what is usually termed the cellular hypothesis there have been presented both rationalizations and experimental evidence to support a humoral concept, namely, the intravascular formation of toxins which in turn produce the symptoms. Wells (143) has stated that there are no pathological changes in anaphylactic shock that are inconsistent with the possibility that some poison similar to the alkaloidal poisons could be concerned. Vaughan and Wheeler (129, 130) demonstrated that from almost any protein a highly poisonous substance can be produced by cleavage under suitable conditions and that this toxic fraction produces effects in animals closely resembling anaphylactic shock. Biedl and Kraus (17) pointed out that the intravenous injection of peptone solutions produced reactions virtually indistinguishable from anaphylactic shock, and Dale and Laidlaw (31) reported that the pharmacological effects of histamine, a derivative of histidin, are strikingly similar to those seen in anaphylaxis. Correspondingly a number of workers had reported apparent exceptions to the statement that a latent period is

essential for passive sensitization and to the statement that simultaneous injection of antigen and antibody into a normal animal does not provoke anaphylactic symptoms. Reference to these exceptions will be made later in the section devoted to anaphylaxis in the rabbit.

There was thus an attractive if not very solid basis for the theory that when a protein antigen is injected the second time it is split into toxic degradation products such as Vaughan's poison, peptone or histamine by the proteolytic ferments which formed as the result of the first injection. As early as 1908, however, Wells (142) had pointed out the extreme improbability of the view that the minute amount of egg albumin that suffices to produce almost immediate symptoms in a sensitized guinea pig could in the short time available undergo proteolysis with the liberation of an adequate amount of toxic degradation products. The validity of this criticism was such that it was necessary either to discard the toxin theory or look elsewhere for its origin. Jobling and Petersen (67) evolved the theory that the union of antigen and antibody in the blood somehow removed from the sphere of action the natural antitryptic constituent of the plasma so that the protease, which it normally holds in check, becomes effective, digesting the proteins of the plasma itself with the formation of toxic cleavage products. While avoiding the quantitative embarrassment involved in considering the antigen as the matrix from which the toxin is derived, this theory had an equally difficult time factor with which to contend and no substantial evidence for it was forthcoming.

Many workers have assumed that the cellular theory and the various humoral theories were mutally exclusive so that as the evidence accumulated that the reaction took place in or on the fixed tissue cells, this evidence was construed as contradicting the humoral or anaphylatoxin hypotheses. The pure cellular theory, however, has no satisfactory explanation for the mechanism by which the antigen-antibody reaction produces the phenomena of anaphylactic shock. When Simonds (120) pointed out that the dominant symptoms of bronchospasm in the guinea pig, circulatory failure in the dog, and cardiocirculatory failure in the rabbit, could all be harmonized on the basis of a spastic contraction of strategically located smooth muscle (in the bronchioles of the guinea pig, in the hepatic veins of the dog, and in the pulmonary arterioles of the rabbit) a distinct step was made in demonstrating that the fundamental reactions in all animals were probably identical. There still remained, however, the question of how the antigen-antibody reaction made the smooth muscle contract, and in addi-

tion how were the phenomena of capillary dilatation and permeability, increased secretion of various glands, changes in the coagulability of the blood, etc., to be harmonized and explained? The crux of the problem from a mechanistic point of view would seem to be the determination of whether the antigen-antibody reaction per se (by producing disturbances of colloidal equilibrium, intracellular precipitation analogous to that seen in vitro, etc.) suffices to explain the varied symptoms, or whether there are in fact any anaphylactic toxins specifically related to the reaction and adequate to account for the symptoms.

Physiological studies of the reactions in the dog, the guinea pig and the rabbit have yielded answers to some of these questions and these observations will be discussed separately in that order although of course there has been no corresponding sequence in their experimental development.

*Anaphylaxis in the dog.* The analysis of the anaphylactic reaction in dogs began with the work of Richet (106). He used water extracts of the tentacles of sea anemones, actinia and mussels, and also a vegetable toxalbumin, crepitin. Although all these extracts were poisonous, and in adequate dosage caused death, Richet noted that sublethal doses, which produced only mild symptoms in normal dogs, would produce violent symptoms and death when injected intravenously into dogs which had received a similar injection two or three weeks previously. A possible cumulative action of the poison was ruled out by the observation that reinjections at intervals of three to five days produced only a moderate effect. The symptoms observed were dyspnea, vomiting, salivation, general weakness, diarrhea and a marked fall in blood pressure. Biedl and Kraus (17) as well as Arthus (6) extended these observations. Biedl and Kraus reported that the fall in blood pressure accompanied and paralleled in degree the severity of the other symptoms. They noted also that while anesthesia prevented the vomiting, diarrhea, etc., it did not prevent the fall in blood pressure, from which they concluded that these reactions were probably a sequel to and less important than, the vascular reaction. They reported that the fall in blood pressure was not due to a weakened heart, but was due to a peripheral vasomotor paralysis. Their evidence for this conclusion was that they found epinephrine to be ineffective in restoring the pressure while barium chloride was much more effective. Eisenbrey and Pearce (51), by direct registration of cardiac activity, and Robinson and Auer (108) by electrocardiographic studies confirmed the conclusion of Biedl and Kraus that the fall in blood pressure was due to vascular and

not cardiac influences. Pearce, Karsner and Eisenbrey (99) demonstrated that with the decrease in arterial pressure there was a decrease in the volume of the kidney, intestine and spleen, while there was a large accumulation of blood in the liver.

Manwaring (82) reported that ligation of the aorta and vena cava above the diaphragm prevented the development of anaphylaxis in dogs and that therefore some infra-diaphragmatic organ was essential to its occurrence. As removal of the intestines, stomach, kidneys, adrenals and spleen had no effect, while short circuiting the liver out of the circulation did prevent the reaction, he concluded that the acute anaphylactic reaction in dogs was an explosive autointoxication of hepatic origin. He performed some cross circulation experiments between anaphylactic and normal animals and as a fall in blood pressure occurred in the normal animal when the anaphylactic reaction was induced in the sensitized animal, he concluded that some blood borne toxic agent, apparently of hepatic origin, was concerned in the reaction.

That the integrity of the liver is indispensable for the development of the complete symptomatology in the dog was further substantiated by the observations of Voegtlin and Bernheim (131) who failed to get anaphylactic shock in dogs after the production of an Eck fistula and ligation of the hepatic artery, by Denecke (35) with similar experiments, and by Simonds and Brandes (121) who found that anaphylactic shock did not occur in dogs when the antigen was injected while the circulation through the liver was prevented by occlusion of the hepatic veins, but restoration of the circulation through the liver permitted the reaction to develop. While some anaphylactic manifestations can be induced in the dog which are not apparently dependent upon the liver (34, 136) it has been generally accepted that the major manifestations of shock in the dog are associated with a hepatic reaction.

Weil (140) and Weil and Eggleston (141) failed to confirm Manwaring's experiments which had seemed to indicate the liberation from the liver into the circulation of a vasodepressor substance. In the first place they claimed that cross circulation experiments would of necessity be inconclusive, since it would be obvious that a marked fall in blood pressure in the anaphylactic animal would result in the normal animal becoming partially exsanguinated by bleeding into the anaphylactic animal until their respective pressures were equalized. Secondly, they transfused blood obtained from anaphylactic dogs as well as blood to which antigen had been added and perfused through the liver of a sensitized dog, into normal dogs and reported that such experiments gave

negative results. They concluded that Manwaring's experiments were in error and that no circulating toxin related to the anaphylactic reaction was to be found in anaphylactic dogs. Weil believed that the impounding of blood in the liver mechanically produced by a swelling of the liver cells was adequate to account for the fall of blood pressure in dogs. In 1925 Manwaring, Hosepian, O'Neill and Moy (83) vascularly anastomosed the hind quarters of a normal dog to a sensitized animal and noted that the urinary bladder and intestine of the normal animal contracted when anaphylactic shock was induced in the sensitized animal. Since this could not be explained by the nonspecific effect of the fall in blood pressure of the donor animal it seemed necessary to conclude that some smooth muscle stimulating agent had been elaborated. This experiment did not provide much opportunity for identifying the smooth muscle contracting substance. However, Manwaring, Reeves, Moy, Shumaker and Wright (84) repeated this experiment using the hind quarters of an immunized animal and as they got negative results with this experiment they concluded that the active substance was a sort of secondary antigen, presumably of protein nature, to which the immunized tissues were unresponsive.

That histamine met the requirements for an anaphylactic poison, at least in so far as smooth muscle stimulation, vasodepression, glandular stimulation, dilatation of capillaries, etc., were concerned, had been indicated since the initial studies of the pharmacology of this substance by Dale and Laidlaw. The dramatic suddenness of the onset of the symptoms in anaphylactic shock prompted, however, a hesitancy in believing that adequate histamine could be formed by ferment action from any protein source within the short time elapsing after the injection of antigen and the development of the symptoms. The observations of Lewis (76) on the cause of various capillary reactions occurring in the skin in response to irritations of various sorts led him to the conclusion that they were produced by a histamine-like substance which existed preformed in the living epidermal cells. While histamine had been found in various tissues by a number of workers it was not until 1927 that Best, Dale, Dudley and Thorpe (16) established beyond reasonable doubt that normal living tissues contained histamine, thereby disposing of the argument that it was formed postmortem by bacterial action or autolysis. As a consequence of these findings Dale (28) restated the anaphylatoxin hypothesis, considering the similarities in the symptomatology produced by histamine and by the anaphylactic reaction adequate for the identification of the anaphylatoxin as his-

tamine, and considering the source of the latter as the tissues themselves and not the antigen or plasma proteins, etc.

With attention thus directed to the tissues, Watanabe (135) reported some analyses of the histamine-equivalent of various tissues from normal, sensitized and shocked dogs. He found the liver histamine of 3 sensitized dogs approximately four times as great as that of 4 normal dogs and of 4 recently shocked animals, while he found no changes in the histamine content of other organs. Although Watanabe recognized the temptation to conclude that these findings indicated a discharge of histamine from the liver into the blood during the anaphylactic reaction he was content to call attention to the fact that marked changes occur in the histamine content of those tissues which are concerned in the anaphylactic reaction. He appreciated the marked variations in liver histamine that are to be found in a series of animals and concluded that until it was shown that the marked rise in liver histamine apparently resulting from sensitization was shown to be a regular and specific change and also until it was shown that a decrease was equally regular and specific for the shock reaction, one could not conclude from his findings that there was a liberation of a histamine-like toxin during anaphylactic shock. It is to be noted that he did not follow the changes in liver histamine in any one animal but assumed that group results reflected such changes.

In 1911 Calvary (19) had reported that anaphylaxis in dogs is accompanied by a marked increase in the flow of lymph from the thoracic duct. This was confirmed by Petersen and Levinson (101) and by others. Simonds and Brandes (122) reported that mechanical obstruction of the hepatic veins, which produces a congestion of the liver similar to that seen in anaphylaxis, likewise produces an increase in thoracic duct lymph. Dragstedt and Gebauer-Fuelnegg (42) were prompted by these observations together with the evidence that the anaphylactic reaction in a dog is predominantly a hepatic reaction, to look for an anaphylatoxin in the thoracic duct lymph. They examined the lymph of anaphylactic dogs and found a smooth muscle stimulating agent which was not present normally and which rapidly appeared as shock developed. They (59) investigated the nature of the active substance and reported that it was a dialyzable crystalloid of basic properties, stable to boiling with acid, and inactivated by condensation with diazotized sulfanilic acid. It contracted the guinea-pig intestine, but not that of the mouse, lowered the blood pressure of the atropinized cat and produced histamine-like wheals in human skin.

They believed this evidence adequate to identify it as histamine. In their first experiments Dragstedt and Gebauer-Fuelnegg did not examine the blood regularly because of Weil's previously mentioned negative experiments, and they reported that only occasionally did they find an active substance in the blood similar to that found in the lymph. In connection with control experiments in which various doses of histamine were injected intravenously into normal dogs so that blood and lymph specimens could be studied in parallel fashion with those obtained from anaphylactic animals, it was observed that histamine disappeared very rapidly from the blood (43). Since in the initial anaphylactic experiments in which the blood was examined, samples were taken at what was assumed to be the height of the reaction, it became apparent that many negative results were probably due to the fact that if histamine had been elaborated with the onset of the reaction it had already disappeared by the time the blood sample was obtained. Consequently Dragstedt and Mead (45) reëxamined a large number of anaphylactic dogs, testing the blood for histamine activity at frequent intervals. They then found histamine activity in the blood regularly whenever an appreciable anaphylactic reaction had occurred. Furthermore, they concluded that it was possible by applying the data on the rate of disappearance of injected histamine to the data on the rate of disappearance of histamine activity from the blood of anaphylactic animals, to ascertain how much histamine had been elaborated in the anaphylactic reaction. They concluded from this comparison that the vascular reaction in anaphylactic dogs can be completely accounted for by the amount of histamine thus computed. Dragstedt and Mead (44) also presented further evidence that the active substance was histamine by showing that it is inactivated by histaminase.

Code (24) using a modification of the method of Barsoum and Gaddum (12) for assaying histamine in blood, has confirmed the above evidence for the liberation of histamine during anaphylaxis in dogs. By making quantitative determinations of the concentration of histamine in the blood during anaphylactic reactions as well as after the injection of histamine he has likewise concluded that the amount of histamine liberated during an anaphylactic reaction is adequate to account for the corresponding vascular reaction. It should be added that Code's results also add to the evidence that the active substance is histamine, since Code and Ing (26), using similar methods, have isolated histamine in crystalline form from a large amount of blood.

Dragstedt (40) and Code (24) have both explained Weil's negative

results. Weil obtained blood from anaphylactic dogs at least 20 minutes or more after the injection of antigen, at which times the bloods would have, in most instances, more or less completely lost their activity. Furthermore he transfused the collected blood into normal animals that were anesthetized, but he did not record the blood pressure in these animals. Consequently, even if an occasional specimen of blood that he used had been active, there was no adequate means for Weil to note any effect.

A striking loss of coagulability of dog's blood during anaphylaxis was noted by Biedl and Kraus and by Arthus. This has been the subject of study by a number of investigators (36, 37, 38, 49, 64, 66, 72, 74, 87, 92, 100, 113, 119, 132, 136, 140, 151) and has variously been attributed to an excess of antithrombin, a diminution of thromboplastin, a deficiency of platelets, the transformation of fibrinogen into an incoagulable tautomeric modification, a decreased prothrombin, decreased fibrinogen, etc. The complexities and changing views of the coagulation process in blood are reflected in these various reports. Parenthetically it may be said that certain investigators (73, 87) have thought that anaphylactic shock was due to a transient intravascular coagulation with fibrin clumps acting as emboli and thus producing the reaction, and that the incoagulability of the blood was a sequel to the coagulative phase. Eagle, Johnston and Ravdin (49) demonstrated that the incoagulability of the blood cannot be accounted for by a deficiency of fibrinogen, a platelet deficiency or a prothrombin deficiency, while they found a very marked increase in the anti-thrombic activity of the plasma. Waters, Markowitz and Jacques (136), making use of the observation of Chargaff and Olson (20) that protamine combines quantitatively with heparin, tested blood from anaphylactic animals and reported a marked increase in the heparin titre. Recently Jacques and Waters (66) have reported the isolation of crystalline heparin from the blood of anaphylactic dogs, thus completing the proof that it is the cause of the incoagulability. There is no reason to suppose that it contributes to any of the vascular or other symptoms (Best, Cowan and Maclean, 15). It is interesting to note that Quick (104) on the basis of an indirect method, came to the conclusion that heparin was the cause of the incoagulability of the blood occurring in peptone shock, which, as has been mentioned, is virtually indistinguishable from anaphylactic shock in its symptomatology.

Presumably in dogs, all or the major portion of the heparin comes from the liver. Weil (140) demonstrated that if antigen is added to the blood

of a sensitized dog no change in coagulability occurs, but if this blood is then perfused through the liver from that animal it will then become incoagulable. Similarly various observers have reported that the injection of antigen into a sensitized dog whose liver has been extirpated or shunted out of the circulation, does not affect the coagulability of the blood.

*Anaphylaxis in the guinea pig.* First known as the Theobald Smith phenomenon, the anaphylactic reaction in the guinea pig was extensively studied by Otto (95, 96) and by Rosenau and Anderson (111). These investigators described the general character of the reaction and clearly defined the circumstances necessary for its occurrence. After the intravenous injection of antigen into a sensitized animal, there is a prodromal period of $\frac{1}{2}$ to 3 minutes during which the animal usually sneezes, scratches its nose, becomes restless, discharges urine and feces, becomes weaker and lies down; acute respiratory difficulty then develops, and death from asphyxia may follow in 2 to 10 minutes. Intraperitoneal or subcutaneous injection of the antigen is much less certain, but may produce death with similar symptoms. The first analysis of the physiological mechanism involved was that of Auer and Lewis (9, 10). They demonstrated that the respiratory difficulty and asphyxia were due to a swiftly developing stenosis of the bronchioles and that in spite of violent respiratory efforts the animal was unable to effect any air exchange. They demonstrated that this bronchial reaction occurred in animals which had been curarized, whose vagi had been cut, or whose spinal cord, medulla and basal brain had been destroyed, indicating that the reaction occurred peripherally. The observations of Auer and Lewis have been confirmed by numerous workers and the demonstration by Dale (27) that the bronchospasm can be reproduced with the isolated, perfused lungs corroborated their conclusion that it was due to a direct action on the muscular walls of the bronchi and not due to a central or reflex nervous reaction. Auer and Lewis (9, 10), Anderson and Schultz (4), and Loewit (77) have recorded the systemic blood pressure in anaphylactic guinea pigs and report that there is an initial moderate rise followed by a gradual fall, such as occurs in asphyxia from any cause. It is thus generally accepted that the predominant reaction is pulmonary and that the circulatory effects are secondary in importance.

With the paramount reaction in the guinea pig clearly defined as a smooth muscle contraction, independent of nervous influences, it was but natural to look for smooth muscle stimulating toxins as important

factors in its genesis. Disregarding the various experiments purporting to prove the formation of an anaphylatoxin as the result of the mixing of antiserum and antigen, the first experimental search for such a substance in the intact anaphylactic guinea pig was, so far as the reviewer is aware, that of Hirschfeld and Hirschfeld (63) in 1912. These investigators tested the blood serum and plasma of anaphylactic guinea pigs by adding them to the fluid used to perfuse the vessels of the frog by the Trendelenburg technic. They reported that a vaso-contracting substance was found that was apparently liberated during the shock reaction. They commented upon the vagaries of its appearance, upon the experimental difficulties involved in studying the guinea pig's blood during different phases of the shock reaction and suggested that similar experiments should be done on the dog. Apparently neither their experiments nor their suggestions were followed up. In 1926 Alexander, Holmes and Becke (3) reported some experiments that might be interpreted as implicating tissue anaphylatoxins of a smooth muscle stimulating character. They reported that when the antigen to which a guinea pig had been sensitized was ground in a mortar with the lung or liver of that animal and then added to the bath in which the uterus was suspended, in many instances they obtained a greater contraction than when the antigen alone was used. They offered no explanation of their results and they apparently did not realize the possible implications as they did not perform the obvious control experiments of mixing antigen with corresponding tissues from a normal animal and of testing both types of tissue-antigen mixtures on a normal smooth muscle preparation.

Watanabe (134) and Hosoya and Watanabe (65) approached the problem from the direction suggested by Dale's theory mentioned previously. They determined the amount of histamine-like substance in the lungs and other tissues of normal guinea pigs, of guinea pigs that had just received the first or sensitizing injection of antigen, of guinea pigs that had received the sensitizing injection about 12 days previously, of guinea pigs that had just received the second or shocking injection of antigen, and of guinea pigs that had been sensitized from 30 to 100 days previously. They reported that there were no significant changes in any tissues except the lungs. They reported that the lungs of guinea pigs that had been sensitized 12 days previously had a very high content of histamine while that of similar pigs that had just received the shocking injection of antigen was very much less. They refrained from concluding that this evidence indicated a release of histamine during

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anaphylactic shock because they found similar low histamine values in the lungs of guinea pigs examined after the first injection which had not produced a reaction, and because they found that the guinea pigs which had been sensitized 30 to 100 days previously showed normal values of lung histamine although presumably they were just as sensitive as the 12 day animals. Rigler (107) previously had examined the pooled livers and the pooled lungs of normal guinea pigs for their content of histamine-like activity and compared the results with the corresponding tissues of guinea pigs that had been sensitized 14 days previously. He found no appreciable difference between the normal and the sensitized animals. Likewise Daly, Peat and Schild (32) failed in 1935 to confirm Watanabe's findings. Mackay (81), however, pointed out that the histamine equivalent content of guinea pigs' lungs was so variable (from 7 to 88 mgm. per kilogram of tissue) that definite evidence of either a rise or fall in lung histamine would be difficult to establish by these methods, unless very large numbers of animals were used.

In 1932, however, Bartosch, Feldberg and Nagel (13) approached the problem from a different angle. They used the isolated, perfused lungs of sensitized guinea pigs and found that a histamine-like substance was liberated into the perfusate after the addition of antigen to the perfusion fluid. The identification of the active substance as histamine was indicated in that both were methyl and ethyl alcohol soluble, both lowered the blood pressure of the atropinized cat and both stimulated the secretion of epinephrine by the adrenal glands. Wachstein (133), Daly, Peat and Schild (32), Ungar and Parrott (127), Ungar, Parrott and Levillain (128), and Schild (112) have confirmed these observations. Daly, Peat and Schild amplified the evidence that the active substance was histamine, and Schild furnished evidence that a number of tissues other than the lungs could also liberate the active substance upon the addition of antigen.

Simon and Staub (123) were apparently the first to detect increased quantities of histamine in the blood of intact guinea pigs. They used the assay method of Barsoum and Gaddum and employed guinea pigs that were sensitized to, and shocked by, arsenobenzene. This observation was confirmed by Code (24), using serum sensitized animals. He found an increase in the blood histamine of anaphylactic animals up to thirteen times the normal values. He also demonstrated that this increase was not due to the coincident anoxemia as normal animals allowed to breathe nitrogen showed no such changes. He estimated that if one could assume that the blood concentrations obtained were the

result of the discharged histamine being distributed in an amount of fluid represented by 60 per cent of the body weight, the amount of histamine apparently liberated was enough to approach fatal doses and therefore obviously of definite significance in the genesis of the symptoms. Actual identification of the active substance in guinea pig blood as histamine has not been made and is probably impossible because of the small amounts available. The chemical and pharmacological evidence is, however, so consistent and so extensive that it may be considered as adequate.

Went and Lissak (146) reported in 1936 that the isolated, perfused heart of the sensitized guinea pig shows a slowing and arrhythmia when antigen is added to the perfusion fluid. This effect was prevented by atropine and was not produced by histamine. As adding physostigmine to the perfusion fluid did not enhance the effect they assumed that choline itself and not an ester was probably concerned. In a subsequent study (147, 148) they reported that a determination of the choline content of the heart muscle before and after an anaphylactic reaction indicated a decrease in the amount of choline in the heart muscle and also that acetylation of the perfusion fluid obtained as before led to the formation of a substance having an acetylcholine type of action on the frog heart and on the leech. Martin and Went (86) likewise reported that the isolated heart of a sensitized animal is more sensitive to the effects of choline than is the heart of a normal animal, concentrations of 1 to 20,000 being effective in the former, while concentrations of 1 to 5,000 are necessary to produce effects in the latter. They concluded that there was both a liberation of choline during the anaphylactic reaction and an increased sensitivity to its effect. The evidence for the former appears to be better than for the latter, but as yet neither observation has been confirmed. The observations of Auer (7) on the protective effect of atropine against anaphylactic shock in the guinea pig might be considered to lend some support to the thesis that choline plays a contributory rôle to that performed by histamine. The extent of its participation in the anaphylactic reaction appears most likely, however, to be limited to the comparatively mild cardiac effects.

There is often comparatively little change in the coagulability of guinea pigs' blood during anaphylactic shock, although occasionally it may become incoagulable. It has not yet been determined whether heparin is concerned in this effect, but the presumption would be that it is.

*Anaphylaxis in the rabbit.* The first experimental study of anaphy-

laxis in the rabbit was made by Arthus (5) although Flexner (54) had witnessed and described the reaction considerably earlier. In this animal the anaphylactic reaction reveals itself either as a local or a general manifestation, depending upon the route of administration of the shocking dose of antigen. The local reaction was first described by Arthus and is referred to as the Arthus phenomenon. When the shocking injection is made subcutaneously there may result infiltration, edema, sterile abscess or gangrenous slough at the site of the injection. Some further reference to this reaction will be made later. When the reinjection of antigen is made intravenously the general reaction is seen. The respiration quickens, the animal sinks upon its abdomen, feces and urine are usually expelled, a fleeting hyperemia followed by anemia of the ears may be witnessed, the heart beat becomes feeble, agonal convulsions may develop and the animal may die within a few minutes. The carotid blood pressure falls greatly, this fall being sometimes preceded by a temporary rise. It is to be noted that the respiration does not become dyspneic as it does in the guinea pig and also that a very much smaller percentage of rabbits succumb than is the case for guinca pigs. Auer (8) and Scott (117) demonstrated that the bronchospasm occurring in the guinea pig did not develop in the rabbit. Auer noted the marked dilatation of the right heart and ascribed the circulatory effects to cardiac failure. Scott on the other hand, noted an obstruction to the flow of blood in the inferior vena cava and believed that capillary dilatation was a major factor in the circulatory effects. Airila (2), Coca (22), Drinker and Bronfenbrenner (48), and Grove (60) have reported convincing evidence that cardiac failure is not a primary factor but that it is secondary to a pronounced obstruction to the flow of blood through the pulmonary circuit. Coca found that during anaphylactic shock the rabbit's lung develops a greatly increased resistance to the passage of saline perfusion fluids. Since embolism and thrombosis can be excluded, he concluded that the increased resistance must be due to a spasm of the arterioles. That the same obstruction occurs in intact animals was inferred by Airila and by Drinker and Bronfonbrenner as they demonstrated a marked rise in the pulmonary artery pressure which persisted until cardiac failure supervened. Histological studies have shown that the pulmonary arterioles of the rabbit present a remarkable degree of muscular development, which appears to be consistent with this assumption, and it may be said that this explanation of the major manifestations of anaphylactic shock in the rabbit has met with general acceptance. More recently Abell and Schenk (1) have made

microscopic observations of the behavior of the smaller blood vessels and blood elements of the rabbit during anaphylactic reactions. They observed the vessels in the rabbit's ear, but there is no reason to doubt that similar reactions occur throughout the circulation. They noted that the injection of antigen provoked both a marked contraction of the arterioles, which in some instances would completely obliterate the lumina, and a marked stickiness of the leukocytes which resulted in clumps of cells adhering to the vascular endothelium and in some instances leukocytic emboli were formed which blocked the circulation in the capillaries and veins. This reaction is undoubtedly the explanation for the observation of Webb (137) who had called attention to the marked aggregation of leukocytes in the capillaries of the lungs of anaphylactic animals, as well as for the leukopenia of the peripheral blood. Dragstedt, Ramirez and Lawton (46) have recently reported that the perfusion of blood to which antigen has been added through the lungs of a sensitized rabbit results in a rapid reduction of leukocytes in the outflowing blood. It seems very likely that this leukocytic reaction may contribute to the effects of the arteriolar spasm in obstructing the flow of blood through the lungs.

That the circulatory reactions in the anaphylactic rabbit are consistent with the pharmacological effects of histamine was indicated by the observations of Dale and Laidlaw (31), Bally (11) and more recently by Rocha e Silva (109). Rose and Weil (110), however, reported that the blood histamine in rabbits is definitely reduced during the anaphylactic reaction rather than increased as it is in the dog and the guinea pig. This has been confirmed by Dragstedt, Ramirez and Lawton. At first glance this would appear to exclude histamine as a mediating agent of the reaction in this animal. Barsoum and Gaddum (12) and Code (23) have demonstrated that rabbit's blood normally contains an extraordinarily large amount of histamine and have presented evidence that the major portion of this is contained in the leukocytes. Assuming that the rabbit's blood, as a tissue rich in histamine, might react as does the histamine rich liver of the dog and the histamine rich lung of the guinea pig, Katz (68) has recently reported that when antigen is added to the blood of a sensitized rabbit in vitro, there is a striking shift of histamine from corpuscular elements to plasma. This has likewise been confirmed by Dragstedt, Ramirez and Lawton who reported in addition that the reduction in the blood histamine in an intact animal is associated with and probably due to the withdrawal of leukocytes from the circulating blood. The amount of histamine that

is liberated from cells to plasma is of such an order of magnitude that if the same reaction occurred in vivo as it does in vitro, it is justifiable to conclude that it is significant in the symptomatology in the rabbit. If the lungs of a sensitized rabbit are perfused with saline solution to which antigen is added there is apparently some histamine liberated from this tissue source (46, 69). It is, however, notably less than that obtained in similar experiments with guinea pigs, and in the intact animal is apparently insufficient to compensate for the histamine withdrawn from the blood by the imprisonment of the leukocytes. There is substantial reason, therefore, for believing that histamine does play a rôle in the anaphylactic reaction of the rabbit and that the fall in the blood histamine is the consequence of the reactions that have made its liberation possible and provided it with its opportunity to act.

There is evidence to indicate either that the leukocytes of the rabbit can be passively sensitized by antiserum without the necessity of any latent period, or that the leukocytes irrespective of any sensitization are injured by the reaction between circulating antibody and antigen. Dragstedt, Ramircz, Lawton and Youmans (47) have found that the simultaneous addition of antiserum and antibody to normal rabbit's blood leads to the same discharge of histamine from cells to plasma as occurs when antigen is added to the blood of a previously sensitized rabbit. They have also found that the simultaneous addition of antiserum and antigen to normal rabbit's blood, used to perfuse the lungs of a normal rabbit, leads to the same withdrawal of leukocytes and of histamine that occurs when antigen is added to the blood of a sensitized rabbit that is perfusing the lungs from that animal.

These observations seem to be of considerable significance in explaining some of the apparent discrepancies between the anaphylactic reaction in rabbits and that in the guinea pig and dog. For example, Friedemann (57) and Scott (117) both reported that no latent period is required for the passive sensitization of the rabbit. Similarly Scott and Opie and Furth (93, 94) have described a reversed anaphylaxis in the rabbit, i.e., a reaction occurring in an animal in which antigen has been injected before the antibody. There have been attempts to explain these reactions as being anaphylactoid in character, but this explanation is inadequate to explain why similar results do not occur in other animals with equal facility. Also it has been reported that the refractory or immune state can not be achieved in the rabbit. Guinea pigs and dogs that have been repeatedly injected with antigen may reach a condition such that the reinjection of antigen after a suitable interval does not provoke an

anapbylactic reaction. The common explanation given for tbis is based upon tbe experiments of Weil and of Dale and Laidlaw and is to the effect that an abundant circulating antibody can combine with and tbus protect the fixed tissue cells from the antigen. The absence of a refractory state in the rabbit in spite of highly abundant circulating antibody is consistent with the view that the fixed tissue cells are of less importance than tbe circulating tissue cells in the genesis of the anaphylactic reaction in this animal. Finally the reviewer is impressed with the satisfactory explanation that these findings give to the experimental facts that the Arthus phenomenon is much more readily produced in the rabbit than in other laboratory animals. The subcutaneous injection of antigen into a sensitized animal, as is well known, leads to the aggregation of large numbers of leukocytes. These cells, richly laden as they are with histamine, by discharging more or less of tbeir content can add the effects of this agent to those of the agglutinating cells per se.

Wenner and Buhrmester (145) have reported that acetylcholine can be detected in rabbit's heart blood during anapbylaxis, but Ratnoff (105) could not confirm their findings. No examination for choline has apparently been made, but the absence of much protective effect by atropine against the anaphylactic reaction in the rabbit suggests that it would have at most only a minor rôle.

Delayed coagulation of rabbit's blood is rather regularly seen in anaphylaxis, although tbe blood does not often become completely incoagulable. Eagle, Johnston and Ravdin (49) have demonstrated that such blood has a high antithrombic activity. If this is due to heparin as it is in tbe dog it would appear from an early observation of Auer (8) that this does not come entirely from the liver as he reported that the usual change in coagulability can be obtained in rabbits ia which the liver has been shunted out of circulation.

*Anaphylaxis in other animals.* The anaphylactic reaction has been observed in a variety of animals but has not been studied extensively enough in any of them to permit of conclusions either as to the important physiological mecbanisms responsible for the symptoms or to the question of whetber tissue anaphylatoxins may play a rôle in the genesis of the reactions. Anaphylaxis has been studied in the cat by a number of workers (18, 48, 50, 82, 114). Cats appear to be rather difficult to sensitize and in addition many foreign proteins produce such marked effects without the necessity of a previous sensitization that there are some experimental difficulties involved in analyzing the reactions in these animals. They show a marked fall in systemic blood pressure,

with some rise in pulmonary artery pressure, analogous to the effects of histamine. The blood may become incoagulable. There is apparently less participation by the liver in the cat reaction than for the dog and less participation by pulmonary vessels in the cat than for the rabbit. No search for anaphylatoxins has been made in this animal.

Hanzlik, Butt and Stockton (61) have studied the anaphylactic reaction in pigeons and have described a contraction of the circular muscle of the crop associated with a relaxation of the longitudinal muscle, which effects they were able to duplicate with histamine.

Anaphylaxis in the rat has been studied by quite a number of investigators (53, 70, 78, 85, 97, 103, 116, 118, 125, 126, 148, 150) and there has been considerable dispute as to whether or not bona fide anaphylactic reactions can be induced in this animal. The rat is peculiarly tolerant to histamine as well as to anaphylaxis. There is evidence that adrenalectomy markedly reduces this tolerance to both. Suden (126) has shown that the systemic reaction in the rat (fall in blood pressure—mild uterine contraction) is similar to that produced by histamine. No direct evidence to implicate tissue anaphylatoxins has been presented.

Various workers have observed anaphylactic reactions in horses and cattle. In general the symptoms, dyspnea, salivation, edema, collapse, etc., are similar to the reported effects of histamine in these animals. Code and Hester (25), however, have reported a fall in the blood histamine of both the horse and the calf during anaphylactic shock. It may well be that some such mechanism accounts for this decline as has been described for the rabbit. In fact Code and Hester state that a centrifuged specimen of control horse blood yielded a well defined layer of white cells while a similar specimen of anaphylactic blood yielded but a thin film, suggesting the disappearance of the white blood cells during the reaction. Since their findings indicated that 65 to 85 per cent of the blood histamine is contained in the white blood cells, it seems logical to conclude that the decrease in blood histamine was associated with the leukopenia. It would therefore be interesting to determine whether the blood cells of the horse react as do those of the rabbit to the addition of antigen.

There is a considerable amount of literature which has not been cited that may be said to provide circumstantial support to the thesis that an anaphylatoxin of the nature of histamine is concerned in the anaphylactic reaction. It consists of reports purporting to prove that a number of immunologically non-specific substances such as anesthetics, adrenal cortex extract, thymoxyethyldiethylamine, atropine, epinephrine, etc.,

protect animals against anaphylactic reactions in about the same degree that they protect against histamine intoxication. While these reports are interesting and of considerable significance, it would carry this review too far afield to consider them here. Furthermore, with the anaphylactic reaction disclosed as a chain reaction (41), involving the antigen-antibody reaction, the discharge of anaphylatoxins, and the subsequent production of effects by these agents, it is apparent that the reaction may be modified or attenuated in a number of ways. Thus without the necessary proof of where or how a nonspecific agent acts it is impossible rightly to conclude from the mere fact that it does modify the reaction that it has done so by virtue of a specific antagonism to histamine, for example. Similarly there are a number of reports indicating that various substances such as glucose, sodium chloride, glycerine, calcium salts, etc., (see Hill and Martin, 62) may modify or prevent the anaphylactic reaction while they do not prevent the effects of histamine. It is equally invalid to conclude from such evidence that histamine is of no consequence in the anaphylactic reaction.

It seems appropriate, however, to mention and discuss briefly some of the arguments which have been raised from time to time to challenge the relation of histamine to the anaphylactic reaction. One of the oldest arguments apparently arose from the observation of Lura (80) that sensitized guinea pigs which have become desensitized or anti-anaphylactic to the antigen do not show any desensitization to histamine or that an injection of histamine does not desensitize the animal to the antigen. However, as Wells (144) has pointed out, we would not expect histamine to desensitize if it is the product of the antigen-antibody reaction, since it is this reaction alone that is prevented by desensitization. Smith (124) has reported that quinine augments the susceptibility of sensitized animals to the injection of antigen but does not increase their susceptibility to histamine. This observation, which has not been confirmed to the reviewer's knowledge, might be due, as Wells suggested, to an action that quinine may have upon the antigen-antibody reaction. It certainly presents no serious contradiction to the evidence for the rôle of histamine. One of the commonest challenges to the histamine pathogenesis has been that it failed to explain one of the dominant symptoms, namely, the incoagulability of the blood. As has been discussed, this manifestation is due to another tissue anaphylatoxin, heparin, which may or may not come from the same source as the histamine and may or may not be discharged in parallel amounts. It is obvious that histamine and heparin explain only those symptoms which they do

explain and that such items in the category of symptoms which they fail to account for demand their respective explanations but do not invalidate in any way the part that these agents play. Another argument against the participation of histamine was that histamine does not duplicate the vascular reaction of anaphylaxis in the rabbit. The effects of histamine on the blood pressure of the rabbit vary with the dose as well as with the anesthetic and Rocha e Silva (109) has shown that if anaphylactic reactions of varying degrees of severity are compared with the effects of varying doses of histamine, the conclusion that the one bears the imprint of the other becomes strengthened rather than nullified. Kellaway (70) believed that the isolated uterus of the sensitized rat, since it contracted upon the addition of antigen while it relaxed upon the addition of histamine, contradicted the histamine hypothesis in anaphylactic reactions. Suden (126) has answered this by demonstrating that the reaction of the rat uterus is different in situ from what it is in the bath, and that the reaction in the intact animal in anaphylaxis is similar to that produced by histamine. It should also be added that the addition of antigen to the isolated uterus in a bath should not be expected to produce a histamine effect unless it could and did liberate histamine from the uterus. That antigen can liberate histamine from a large variety of tissues does not prove that it can do so from all tissues and it may well be that the rat uterus in situ reacts to histamine which has been liberated from other tissues, while the corresponding uterus in a bath shows only the non-specific effect of a foreign serum. The isolated plain muscle from a sensitized dog gives no significant response to the specific antigen, yet the plain muscle of the dog is sensitive to histamine and, in addition, a contraction of smooth muscle organs occurs during the anaphylactic reaction in the intact animal.

## SUMMARY

The evidence may be considered conclusive that a tissue liberation of histamine, of heparin, and possibly of choline occurs during the anaphylactic reaction in various animals. In the dog the liberation of heparin can completely account for the incoagulability of the blood and there is no reason to doubt that it may be found in other animals. There is no evidence that it is responsible for any of the other dominant symptoms as large amounts of heparin can be injected into animals without harm. The liberation of histamine can account for practically all of the other effects and there is no well defined prominent symptom demanding an alternative explanation. Such changes as loss of complement, etc.,

are excluded from this statement and may be considered as being related to the antigen-antibody reaction per se. The observations of Went suggest that choline may participate in the reaction in the guinea pig and possibly of course in other animals, although further evidence is necessary to establish this. The leukopenia in all animals can be explained by the mechanisms described by Abell and Schenk in the rabbit, and it is probable that leukocytic emboli may contribute more or less to the vascular reactions produced by histamine. For animals other than the dog, guinea pig and rabbit decisive evidence is lacking, but the symptomatology is nowhere incompatible with the conclusion that similar reactions take place.

The cardinal symptoms of anaphylaxis can thus be explained as being due, in the immediate instance, to an autointoxication by physiologically active substances normally resident in various tissue cells and liberated therefrom by some change in cellular permeability brought about by the antigen-antibody reaction. It is apparent that the tissues concerned may be either the fixed tissues as in the dog or guinea pig, or the circulating tissue as in the rabbit. It is also apparent that the reactions mediated by these anaphylatoxins may occur in the tissues from which they were liberated or in distant organs and tissues. The mechanism by which the antigen-antibody reaction leads to the rather special type of cell injury resulting in the discharge of the agents characteristic to the anaphylactic reaction presents the next challenge in the study of this interesting subject, but there is considerable satisfaction in being able to discard the various esoteric speculations regarding the pathogenesis of the symptoms.

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# ANTIHORMONES

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The present interest in the study of antihormones was precipitated by the experiments of Collip and Anderson (31a), whose foresight led them to study the sera of animals that had become refractory to the action of certain pituitary hormones. They discovered that the serum of an animal resistant to thyrotropic hormone prevented the action of this hormone in a test-animal. This effect was ascribed to what they termed an "*antihormone*", which apparently accumulated in the injected animal's serum in response to the hormone administered.

These results conceivably were what an immunologist would have predicted, for in the various texts of immunology there has accumulated evidence which indicates that specific cytotoxins or cell-destroying antibodies may be produced by the injection of extracts of specialized cells. More specifically Abderhalden (1) has described "Abwehrfermente" as appearing in the blood of experimental animals chronically treated with extracts of endocrine glands.

The discovery of the antagonistic action of sera of hormone-treated animals deservedly aroused much interest among endocrinologists and physicians. Surprisingly enough this degree of investigative enthusiasm apparently has not been shared by the immunologists, whose authorities indeed had predicted the discovery of antihormones. The work thus has not progressed as rapidly as one would desire, because, as will be seen later in this review, certain essential experiments dealing with the immunological aspects of the problem have not been undertaken.

Collip, in his original statements (27), likened the antihormone not to an antibody but to the "chalone" of Sharpey-Schäfer. This pioneer in endocrinology postulated (181) the existence of substances possessing purely inhibitory effects, and he proposed for them the term "chalone", based on a Greek word meaning "to relax". Collip (29) preferred to

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call the inhibitory principle "antihormone" rather than "chalone" on the following grounds:

> . . . In spite of the very logical nature of Schäfer's objection to the use of the word "hormone" to describe all of the internal secretions, both excitatory and inhibitory, the term has been accepted by general usage.
>
> While there may appear at first sight to be an analogy between the substances which I have called "antihormones" and the "chalones" of Schäfer, the theoretical conception of the mechanism of production and the physiological significance of the former were such as to demand the use of a descriptive and distinguishing term.

The reviewer believes that the term "*antihormone*" is the best and most descriptive name offered for the class of substances under consideration. The experimental evidence submitted thus far leads one to classify these inhibitory principles as immune bodies. There is ample precedent for the use of such a word as antihormone in this sense, where it would be comparable to antitoxin or antienzyme, all being antibodies that are produced in animals in response to antigens. The writer herein aims to use the word antihormone in a way that is consistent with immunological terminology, and in this respect differs from Collip whose tendency is to use the word in the sense of a chalone. For instance, Collip (29, 32a, b) has used the term "antithyrotropic hormone" while I have written "thyrotropic antihormone". It would seem permissible to use the term "antigonadotropin" to indicate the antihormone of a gonadotropic hormone, but I would not favor the term "antigonadotropic hormone". This discussion will serve to clarify the terminology used by the reviewer in the following paragraphs.

The problem. The problem of the antihormones has resolved itself into the following parts: What is the nature of the inhibitory principles termed "antihormones"? How does the body produce them? What is their chemical nature? What are their physiological actions, their possible therapeutic uses? Although not all of these questions can be answered, nevertheless much information is available for this review. At first glance the material seems almost hopelessly confused by the number of hormones and the variance of their sources, the wide variety of species of test-animals experimented upon, and the number of different approaches used in the study of the problem. Much of the work is open to one serious criticism, which is that many of the investigators have been too broad in their claims, ascribing far-reaching conclusions to results that actually were narrow in their scope and application.

In order to simplify and to improve the readability of this review I have

commenced each of the longer sections with a summary of my interpretation of the published reports which then are quoted in greater detail. Not every publication has been listed for I had to be content with a selection of representative data on the various aspects of the problem. I have treated the antihormones as a group of substances of essentially similar nature, rather than as a number of separate entities each with special qualities. Should a supplement for this account be desired the reader is advised to consult a recent detailed review of the subject by Collip, Selye and Thomson (29, 32a, b), and briefer summaries by Evans (62), Foa (69), Marrian and Butler (127), Werner (209c), Wintersteiner and Smith (211), and others (70, 163).

Early reports. Prior to 1934, when the first publications on antihormones appeared from the Collip laboratory, there had been performed four types of experiments which have bearing on the present subject. The first dealt with an inhibitor for thyroglobulin, first found by Möbius (139) in the blood of thyroidectomized sheep. Such a preparation, called "antithyreoiden-Möbius", originally made available by the Merck Laboratory, has continued to be of experimental interest, especially in Europe, and will be discussed under the heading "antithyroid principles". Another group of studies dealt with the anti-insulin effects of various pancreatic preparations as shown by studies of the blood sugar values of treated animals. An anti-insulin action was attributed to the impure preparations of insulin. It is not clear from the experiments whether the effects on the glucose values were due directly to a specific chemical inhibitor in the extracts, or to some non-specific reaction of the injected animal such as the secretion of epinephrine. For this reason, these particular experiments and certain others contribute little to the problem save perhaps the knowledge that a concept of inhibitory factors or antihormone was present in the minds of these investigators: Cotte, 34; deJongh, 40; Meyer-Bisch and co-workers, 138; Nobel and Priesel, 142; Sayhun and Blatherwick, 179; Wichels and Lauber, 210; and others.

Investigations more pertinent to the present problem are those dealing with hormones as antigens. With few facts available concerning them antisera to the pituitary early received a fruitless clinical application in hypertension and diabetes at the hands of Legiardi-Laura (107, 108, 109). In 1923, however, Hektoen and his co-workers (90, 91, 92, 93a, b) reported the first of an important series of studies on the precipitin reactions of thyroglobulin. They demonstrated that an animal reacts to injections of thyroglobulin by producing a species-specific immune body (precipitin) and lesser quantities of non-species-specific immune

bodies. The precipitin reactions of pure thyroglobulin were successfully used by Hicks (98) to trace this substance in certain animal fluids. These investigations have been fortified by others with comparable results: Clutton *et al.*, 24, 25, 26; Picado and Rotter, 157, 160; Went *et al.*, 208.

Koyano (104) in 1923 injected ox-pituitary extracts into rabbits, with the stated aim of producing a pituitary antiserum. This preparation was injected into rats, whose adrenals and pituitaries finally showed cytological changes, which will be discussed later. Koyano unfortunately did not study the problem of the species-specificity of his antiserum. Somewhat similar experiments had been carried out by Masay (129, 130), who induced in animals a state akin to myxedema by the injection of a pituitary antiserum.

Despite the foregoing and other suggestions in the literature that crude glandular extracts when introduced parenterally might be antigenic, little heed was paid the fact that the resultant immune body might have physiological actions until the refractory state was observed in animals that had been injected for long periods with the available crude pituitary extracts. These observations came to be made in the following manner:

The refractory state. P. E. Smith's publication (193), *The disabilities produced by hypophysectomy and their repair*, called forth a wave of contributions that established the existence of various "tropic" factors in the pituitary. An avenue was thought to have been opened for the possible reproduction of certain disease syndromes which conceivably could be attributed to an overactivity of one or another of the pituitary "tropic" hormones. Save for one exception (experimental acromegaly with the growth hormone) the long-term injection of pituitary extracts failed to produce the expected results when a heterologous pituitary preparation was injected into an animal. The injected animals developed a refractory state (6, 31a, 71, 97, 113, 117, 118, 119, 120, 121, 131, 133, 182, 185, 193, 203a, 218) whereas in P. E. Smith's original experiments with homologous preparations (i.e., rat pituitaries in rats) the hypophysectomized animals' subsidiary glands were maintained in a normal state.

Discovery of the antisera. Collip and Anderson (31a), while studying the nature of this sort of resistance in animals injected with thyrotropic hormone, noted that the sera protected test-animals from the therapeutic effects of injected thyrotropic hormone. An extract of liver and spleen from the thyrotropic-resistant rats also prevented the

rise in metabolism normally expected from thyrotropic hormone injections, whereas normal rat serum and horse serum had no such effect. Collip and Anderson stated that the thyroid glands of the rats showed the characteristic hypertrophy which normally is found following treatment with thyrotropic hormone alone, and further, that rats whose blood contained the inhibitory substance still responded with a rise in metabolic rate when fed desiccated thyroid. They suggested that these experiments indicated the possibility of a new method for the treatment of clinical hyperthyroidism.

Shortly thereafter other investigations (28, 29b, 30) in the same laboratory established the fact that similar inhibitory effects in several species of animals could be demonstrated for other principles, namely: the ketogenic (15a, b) and the gonadotropic hormones from the pituitary (188e), and for A.P.L. (9, 188d) (the anterior-pituitary-like hormone of the urine of pregnancy).[1] Collip stated that he had evidence for a growth antihormone (28). He also mentioned that similar antagonistic substances may occur spontaneously in the blood of certain patients; here no details were given, save to say that antagonistic substances in the blood of 10 patients occurred spontaneously and not following pituitary therapy.

*Collip's antihormone theory.* In order to explain the remarkable results of these experiments and to provide a working hypothesis for further experiments, Collip (27, 28) advanced his "antihormone theory", which may be quoted as follows: "For each hormone there may be an opposite or antagonistic principle. This antagonist is present in the normal subject but may not be detected until it exceeds in amount the hormonal substance with which it is balanced." This concept received further elaboration as a "*principle of inverse response*" where Collip postulated a system of hormonal check and balance of the endocrine glands, each hormone having its inhibitor.

THE METHOD FOR BIO-ASSAY. There have been a number of different methods used for the bio-assay of the antihormones. The principles followed in most of the reports have been similar to those initiated by Collip and Anderson (31a), viz.: 1, the antiserum is injected at a site different from that for the injection of the hormone; 2, a short period of pre-treatment with antiserum is followed by treatment with hormone while yet continuing the serum. The titer of antihormone activity

[1] The author hereafter for the sake of simplicity will use the abbreviation, "A.P.L.", in place of the following: anterior pituitary-like hormone of the urine of pregnant women; prolan, human chorionic hormone.

customarily has been estimated by determining the minimum dose of antiserum required to inactivate a standard dose of the hormone in the test-animal. Almost every species of the common small laboratory test-animals have been employed depending upon the type of experiment conducted. Zondek and his associates (224) report that rats are most suitable for testing the potency of antisera to A.P.L. It is important in these studies to make careful note of species of test-animal.

THE NATURE OF THE ANTIHORMONE. Collip (27, 28, 29, 32a, b) apparently rejected the notion that these inhibitory substances might be antibodies after he noted the immunological studies of Bachman (8), who wrote the following:

> A.P.L.-treated rabbits yield sera which give weak immunity reactions *in vitro* with solutions of A.P.L. and extracts of male urine, and stronger, but less constant reactions with human serum proteins. At the present time, however, there appears to be no clear connection between these phenomena and the A.P.L.-inhibitory property demonstrable in such sera with biological methods.

This statement of Bachman's cannot be interpreted to mean that the antihormone is not a variety of antibody, and it actually did not wipe away the suspicion of others that the immune processes of the body might play a rôle in the production of antihormones. There appeared, then, in this vein, a series of reports which gave further consideration to, and incidentally a certain amount of support for, an antigen-antibody concept of the antihormone.

THE NUMBER AND VARIETY OF ANTIHORMONES. The injection of pituitary *thyrotropic hormone* or the *gonadotropic hormones of the pituitary or of the chorionic tissues* readily induces in animals a refractory state associated with demonstrable antihormones in the serum. Most of our knowledge of these inhibitory substances is based on observations of the behavior of the thyrotropic and gonadotropic antihormones. However, under suitable conditions other pituitary hormones and certain non-protein hormones give rise to effects which indicate the production of anti-hormones, but relatively little is known about them perhaps because tests for the hormones, which they inactivate, are uncertain of results or are more difficult to perform. Although the antihormones seem to be alike in their behavior, we must not assume that the properties of any one of them are the properties of the group until this has been demonstrated.

Mention should be made of the fact that in addition to the antihormones which have physiological effects, the hormonal preparations may

also give rise to antibodies that have no demonstrable physiological effects (11, 12, 13a, b, 190). Certain other principles which are not antihormones but which may be confused with them will be discussed under the title "anti-thyroid principles". Engel (57, 58, 59) has suggested that the gonadotropic inhibitory principle may be a product of the pineal gland, but what little evidence he has offered has not been supported. Since the thyrotropic and gonadotropic antihormones will receive much consideration later, now a brief description of the other antihormones will serve to present the knowledge which is available concerning them.

Collip (28) assumed that the *growth-hormone* may give rise to an antihormone, because hypophysectomized rats treated with a highly purified preparation failed to maintain satisfactory growth despite continued treatment. Evans *et al.* (61) confirmed these experiments of Collip's, but with crude extracts obtained excellent growth of similar animals. This and other experiences with the growth-hormone, especially the experimental reproduction of acromegaly, leads one to doubt whether an antihormone ordinarily is produced by the growth-hormone. However, final judgment on this and similar situations must be reserved until we are able to study the pure hormone under controlled conditions.

The *ketogenic principle* has a pronounced ability to induce the refractory state, and the antiserum confers similar resistance on test-animals. The refractory animal's own ketogenic hormones may be inactivated (15a, b). Likewise, animals develop a refractory state to the pituitary *adrenotropic hormone* (203a), but little is known about this antihormone.

*Prolactin* preparations also call forth antihormones. Young's (214) summary of his own work here will be quoted, for it may have significance for the thyrotropic hormone as well as for prolactin; viz.:

> 1. Large doses of ox prolactin possessing no detectable thyrotropic activity were administered daily to rabbits and monkeys for long periods. The serum of animals so injected for 12 weeks or more possessed marked ability to prevent the hypertrophic response of the pigeon crop gland to prolactin. 2. The average weight of the young reared by lactating mice which received daily injections of rabbit antiserum was slightly, but probably significantly, less than that of the young of control animals receiving similar injections of normal serum. This was possibly due to partial inhibition of milk secretion in those animals receiving antiserum. 3. Rabbits which had received daily injections of prolactin for 18 weeks exhibited a normal hypoglycaemic response to insulin after the administration of a potent pituitary glycotropic preparation, i.e., they were insensitive to the glycotropic action of the extract. No evidence was found, however, that this resistance to the glycotropic action of pituitary preparations was transmissible to normal animals by the serum of the resistant rabbits. 4. Although the prolactin

used to prepare the sera contained no detectable thyrotropic activity, the antiserum obtained from the rabbits possessed substantial antithyrotropic activity. By thyrotropic activity is meant the ability to induce hypertrophy of the thyroid gland of the immature guinea pig.

The essentials of these experiments have been confirmed by Rowlands and Young (177), and by Bischoff and Lyons (14). Prolactin, apparently, can be precipitated from an extract by incubation with prolactin antiserum, thereby reducing the physiological activity of the extract (Strangeways, 196). This method conceivably might be useful in the purification of extracts.

An antihormone for the *diabetogenic hormone* has been considered as the explanation for results of Dohan and Lukens (43), who injected pancreatectomized dogs with crude anterior pituitary extracts and after a time observed a decrease in the glycosuria. The animals in some respects came to resemble Houssay preparations, and the possibility was entertained that the diabetogenic principle was inactivated by the production of an antihormone in the animals.

Certain *non-protein hormones* have given rise to a state of refractoriness with anti-substances in the serum. Hartman and his associates (89a) have observed that *adrenal-cortical hormone* preparations lost their effect after repeated injections into normal dogs. The serum of dogs resistant to the adrenal-cortical hormone, when injected into other dogs, was found by Toby and Lewis (204) to inhibit the effect of cortin. These antigenic cortical extracts were highly purified, contained very little solid material, were free from protein, and contained practically no nitrogen. These extracts gave precipitin reactions with serum from the refractory but not from normal dogs. There was some evidence which indicated that the antisera had some power to fix complement. More recently Hartman *et al.* (89b, c) determined that crystalline corticosterone itself did not produce the inhibitory effect, but this did occur when the pure product was combined with swine serum before injection. This use of swine serum is a well known "trick" of immunologists whereby a substance not ordinarily antigenic is rendered so by conjugation with an element in the swine serum; the combined substance reacts as the antigen. The substance which confers the specificity but not the antigenicity is termed "*haptene.*" Sulman (197a), as will be seen later, claims that A.P.L. is not a haptene. Swine serum is not the only agent that confers antigenicity on substances which are not antigenic. For example, phenol has been used in this manner to produce antigenic adrenaline (199).

Brandt and Goldhammer (19) have produced complement-fixing antibodies to an *androgen* and an *estrogen* by mixing these substances with swine serum before the injection. In their experiments, however, the hormones were not inactivated physiologically by the antisera, whereas such was the case in Hartman's experiments with preparations of cortin. The injection of estrin alone apparently does not call forth antihormones (37), although this and other sex-hormone substances exert on the gonads and other tissues and hormones an inhibitory effect that might be confused with an antihormone effect (38, 72, 74, 152, 164).

Somewhat different, however, is the well known refractoriness to the repeated administration of the *parathyroid hormone*, for Taylor *et al.* (200) have presented proof that it is not due to an anti-substance in the serum. The sera of their resistant animals failed to give complement-fixation reactions with the extract or to inhibit the action of parathyroid extract biologically. The refractory animals tolerated massive doses of irradiated ergosterol that normally would be toxic in dogs not so treated with the parathyroid extract. Somewhat akin to parathyroid refractoriness is the state Selye (187) has observed resulting from prolonged treatment with estrogen. The refractory state was not transmitted by the serum of the animal.

THE NATURE OF ANTIHORMONES. *1. Hormonal preparations interpreted as antigens.* The foregoing account has described how the antihormones were presented as agents having important physiological actions. At once there was discussion as to their nature, and Collip and his associates proposed that they be looked on as chalones rather than as immune bodies, but he named them "antihormones." Successive reports appeared amply confirming the fact that there are such inhibitory substances, but each report tended to place them in the class of substances called antibodies rather than in the class called chalones.

Lacking pure hormones or pure antihormones the majority of investigators have had to be content to perform experiments devised to show the pattern of antihormone behavior; the facts were then compared with what we know about antigens and antibodies or hormones and chalones. In every important respect antihormones were similar to antibodies. The problem is made doubly difficult by the fact that we do not know the physical or chemical nature of the immune bodies, of which naturally there are a great number. Furthermore, the antihormone may be a variety of antibody that hitherto had been unknown and for which we have no precedents, save those common to antibodies in general.

Although the antihormones tend to appear and to disappear with the precipitins and complement-fixing antibodies, there has been no success in attempts to correlate more definitely these antibodies with the antihormones. Indeed the evidence clearly indicates that the antihormones are not linked with the above types of antibodies. On the basis of the observation that A.P.L. reacts with its antihormone quantitatively rather than by a process of adsorption, the suggestion has been made that this antihormone is a special type of ferment, and not a true immune body. One should not hastily use this evidence because the immunologist probably would tell us that certain immune bodies do react quantitatively with their antigens.

In several other respects the antihormones have behaved as if they were antibodies, viz.: they are not called forth by homologous extracts and do not appear in parabiotic animals with prolonged pituitary hormonal hyperactivity. Neither do they appear when a crystalline hormone (corticosterone) or a highly purified preparation is used. Nor do they appear in animals when the reticulo-endothelial system (the site of origin of immune bodies) is thrown out of function, or is inactive, as it is in the young or in certain states of debility. The antihormones seem to have a humoral action; no fixed tissue, neither the affected subsidiary glands nor the one originating the inactivated hormone, takes a necessary part, save the reticulo-endothelial system which produces the antihormones. Likewise, the changes in species-specificity of antihormones proceed on lines that have precedents in immunology. The finer details of the non-species-specificity of antihormones and the possible bearing of the Forssman or heterophile antigen upon this problem await special study.

One inevitably concludes from the present evidence that the antihormones belong to the class of substances called immune bodies, but they are a variety that has certain unusual characteristics. The hormonal preparations that stimulate their production are therefore antigens. These antigens evidently belong to the group that immunologists term *"complex antigens,"* for the hormone can be separated by suitable methods from the carrier substance, which is antigenic while the hormonal radical is not. Although there exist data indicating that the hormonal portion of the antigen is a haptene, yet at present this cannot be assured; the various hormones may differ in this respect. The data now follow:

Twombly (205) demonstrated that preparations of A.P.L., which had been almost completely inactivated by heat or by ageing, were as

efficient in producing an antihormone as were active preparations. In Twombly's experiments, the antihormone titer increased with the precipitin titer (206). The foregoing experiments were confirmed by de Fremery and Scheygrond (39), who also noted that the serum of non-pregnant mares did not produce antihormone effects when injected into rabbits. Zondek *et al.* (219), however, claim that the A.P.L. is not completely inactivated by boiling, and that the antihormone-producing activity is due to the residue of active hormone.

Werner (209a, b, c) was able to induce in male guinea pigs a refractory state to a thyrotropic preparation made by a sodium sulphate precipitation method from bovine pituitaries. This he was unable to do with an extract equally potent in thyrotropic activity made of bovine preparations by flavianic acid precipitation. The foregoing work of Werner has been confirmed by Cutting *et al.* (35). Werner also showed that the thyroids of the refractory guinea pigs were stimulated by the flavianic acid product, and further, that the serum of non-refractory guinea pigs that had been injected with the flavianic acid preparation had no antihormone effect in test-animals. These experiments were interpreted to mean that the antihormone was produced by a substance associated with the hormone, but not necessarily by the hormone itself.

Eichbaum and associates (49) studied the problem of the antigenic properties of hormone preparations, and came to the conclusion that the complement-fixing antibodies produced by the injection of urinary extracts were due to a urine antigen, and not to the A.P.L. in the urine injected. They found that the antibodies for urine antigens did not react with antigens from homologous blood or skeletal muscle. In another experiment (50) they injected male and female rabbits with A.P.L. and observed in serum, first, precipitin and complement-fixing antibodies, which had no neutralizing effect upon A.P.L.; next, biologically active antihormones appeared; and lastly, after continued injections the precipitin and complement-fixing antibodies disappeared, leaving unimpaired in the serum the inhibitory action. These facts were interpreted to indicate that the antihormone activity is not necessarily affected by the precipitin or complement-fixing reactions. Brandt and Goldhammer (18) have come to similar conclusions.

Somewhat related experiments are those of Gegerson *et al.* (73), who prepared antisera in rabbits by the injection of A.P.L. and of bovine pituitary extracts, and then incubated the antihormone sera with human and bovine serum respectively. The heavy precipitate was removed, but the remaining serum retained its antihormone activity. This indi-

cated that the antihormone is a specific substance unrelated antigenically to the serum proteins. Gustus, Meyer and Dingle (85) also working with precipitin titers were unable to correlate precipitin titer and antihormone activity when they used monkey antiserum to pregnant mare gonadotropic hormones. A number of investigators (41, 42, 135, 153, 155, 159) were unable to correlate the precipitin titer with the antihormone activity of the sera, although some others found evidence that appeared to connect the two (60). Meyer and Wolfe (137), using gonadotropic hormone of pregnant mares, have shown that monkeys produce precipitins and antihormones following injections of small quantities of hormone, but that the precipitins are not responsible for the antigonadotropic action of the sera. Gordon *et al.* (79) used highly purified preparations of the mare chorionic hormone which produced an inhibitory action but no precipitins. The antihormone-producing property of the extract was possibly enhanced rather than decreased by the process of purification (78, 79).

Sulman (197a) has carried out numerous experiments attempting in one particular report to answer the question "does the gonadotropic hormono (A.P.L.)[2] induce antibodies or antihormones?" The claim was made that in rabbits "pure" A.P.L. did not call forth complement-fixing antibodies, whereas crude A.P.L. did do so. "Pure" A.P.L. coupled with swine serum also called forth complement-fixing antibodies, which, however, reacted only with the mixture and not with the A.P.L. alone. From these facts Sulman concluded that the "pure" A.P.L. was neither an antigen nor a haptene, but was some unusual form of protective ferment. *The "pure" prolan of Sulman was prepared by a pharmaceutical firm, and Sulman gives no references concerning the method for preparing the product, and no proof of the purity of the substance.*

Van den Ende (207) has submitted a comprehensive report and discussion on the preciptins in antigonadotropic sera, giving the following summary:

1. Antisera to an active gonadotropic extract of pregnancy urine contain precipitins demonstrable with a variety of urinary extracts, independent of their hormone activity. The amounts of precipitins present could be determined by optimal proportions. 2. Precipitin content and antigonadotropic power showed no constant relationship to each other. 3. Pregnancy urine extracts contain multiple antigens. 4. Absorption of precipitins by an extract of the urine of males resulted also in the removal of all precipitins for urinary gonadotropic extracts, but only partly reduced the biological inhibitory power. On the other

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[2] The insert enclosed in parentheses is the reviewer's.

monkeys with pregnant mares' serum and found the antagonistic effect was highly specific in the antiserum, there being no inhibitory effect toward the hormones of A.P.L., sheep or human pituitaries, when tested in immature rats. Yasuda (212) injected rabbits with A.P.L. and found that the resultant A.P.L. antiserum did not inactivate bovine gonadotropic hormone when tested in rabbits. Sulman (197a) found that antihormone against A.P.L. in rabbits had no inhibitory effect on the gonadotropic hormone of the serum of pregnant mares. Zondek *et al.* (220) report that the antihormone to A.P.L. in rabbits is highly specific; less than 0.5 per cent of the effectiveness being available for a heterologous preparation, whereas 7 per cent of the activity was available for a homologous factor (serum of pregnant women), and the remainder for the specific factor.

On the problem of organ-specificity or of hormone-specificity there is less information available. There appears to be no doubt that one can prepare with the proper treatment an antiserum which inactivates the gonadotropic action of chorionic as well as pituitary hormones. For example, Thompson and Cushing (203b) injected a dog for many months with a sheep pituitary preparation, and the dog's serum inactivated the gonadotropic effects of several species of pituitary glands as well as the action of pregnant mare serum and A.P.L. Likewise a dog injected with pregnancy mare serum inactivated gonadotropic extracts from sheep pituitary glands. Rowlands and Parkes (175b) injected a goat with A.P.L. and found that its serum inactivated the gonadotropic hormone of human pituitary glands. In regard to class-specificity, Parkes and Rowlands (151) have shown that in birds antisera to mammalian thyrotropic and gonadotropic substances are ineffective.

Work on the hormone-specificity of antihormones is hampered by the lack of chemically pure pituitary hormones. One of the few experiments with so-called "pure preparations" is that of Rowlands and Young (177) who found that prolactin antisera, produced by injection of an extract apparently free of thyrotropic activity, had a markedly anti-thyrotropic effect. Here however, there remains the suspicion that a trace of thyrotropic hormone may have been present in the antigenic extract. With the available pure non-pituitary hormones there seems to be no doubt of a strict hormone specificity, when antihormones are successfully produced.

Antihormones for gonadotropic (176) or thyrotropic (194b) hormones apparently develop in man, as they do in other animals, under appro-

priate conditions. Thus far in patients injections have been terminated when the refractory state was noted, and no instances of inactivation of the subject's own pituitary hormones have been recorded. Whether these antihormones are the same as the inhibitory substances which occasionally are found in normal human sera has not yet been determined.

*3. Experiments with homologous preparations.* The data quite clearly indicate that the injection of homologous preparations in animals does not ordinarily give rise to antihormone formation. Whenever the antihormone effect has been obtained with these conditions, the antisera have been weaker in potency than antisera resulting from heterologous preparations. These few exceptions could conceivably be explained by assuming that some of the proteins were denatured in the process of preparing them for injection. This might render them antigenic in the homologous species. The immunologist conceivably might offer another more suitable explanation for these exceptional observations.

The failure of homologous preparations ordinarily to stimulate the formation of antihormones lends strong support to the supposition that the antihormone is a variety of immune body. Certain cleverly designed experiments with parabiotic rats have conclusively proved that an over-stimulation with pituitary hormones can proceed for long periods without any suggestion of the development of the refractory state, or of antihormones. These experiments strongly oppose the chalone concept of the "theory of inverse response." The following will serve to present the material available on the subject of homologous preparations:

Twombly (205, 206) and others (44, 193) were unable to demonstrate antihormone effects in human sera after prolonged parenteral treatment of patients with A.P.L. (human, in origin). Likewise, Rowlands (168) and Thompson (201) were unable to produce gonadotropic antihormones in sheep injected with crude extracts of sheep pituitary glands. These investigators observed a slight augmenting effect in some of their tested sera. Collip (30), on the other hand, noted a weak antihormone effect in the serum of two out of four sheep injected for long periods with a crude, gonadotropic extract of sheep pituitaries. The antisera in these particular animals of Collip's were effective against bovine pituitary, pregnant mares' serum, and possibly hog pituitary extracts. Collip also observed some signs of synergism between some of the sera and the gonadotropic substances.

Supporting P. E. Smith's original contribution, Katzman, Wade and Doisy (101a) were unable to demonstrate refractoriness in female rats

following the daily administration of one or two rat pituitaries for a period of from seven to nine months. The serum of these animals gave no gonadotropic or thyrotropic antihormone effect in test rats or guinea pigs, but did give an augmenting effect. On the other hand, Selye, Collip and Thomson (188e) found that the ovaries of rats receiving daily implants of rat pituitaries for 68 days were normal or subnormal in weight; this fact they interpreted as evidence of a refractory state. Anderson and Evans (4) have claimed that large doses of rat thyrotropic hormone injected into rats gave rise to a thyrotropic anti-substance in the serum of the animals; the effect was detected by a slight depressant action of the serum upon the $O_2$ consumption of guinea pigs receiving a standard dose of thyrotropic hormone.

Even more satisfactory experiments with a *more physiological type of homologous administration of hormones have been those using parabiotic rats* (36, 46, 128, 132). These ingenious studies showed that the gonadectomized parabion caused a continued stimulation of the gonads of the normal or hypophysectomized twin, producing in it a constant state of estrus as a result of the greatly increased output of pituitary gonadotropic hormone from the castrated animal. This condition of continued stimulation was maintained for long periods of time by different methods, and indicated that under these conditions no gonadotropic antihormone was produced in either animal in response to the pituitary hypersecretion. Kupperman *et al.* (105) showed that an antihormone in rabbit serum prevented the action of endogenous gonadotropic hormone secreted by the pituitary gland of a castrated rat in parabiosis with a normal female.

*4. Time of appearance and disappearance of antihormones.* The antihormone production begins in an animal shortly after the first injection of a suitable preparation, and the inhibitory substance accumulates in sufficient quantities in the blood stream to produce a noticeable effect in 7 to 12 days. This effect is the inactivation of the injected extract, producing the refractory state. After 21 to 28 days the serum is highly active in its power to inactivate the antigenic extract in test-animals. After long periods of injections into suitable animals the non-species-specific antihormones appear in sufficient quantities to inactivate the injected animal's own hormones, and at this time the antiserum can be expected to have actions on antigens from species other than the one used for the injections.

After injections of the antigen are stopped the antihormone activity gradually decreases in the serum, so that usually it may not be detected

after 30 days. In one instance it was detected for 2 months after the injections were stopped. A single injection of antihormone in homologous serum may protect an animal from the antigenic hormone for from 8 to as many as 30 days. Mature animals seem to produce antihormones more effectively than young ones; rabbits possibly are more satisfactory antihormone-producers than are other animals. Crude hormonal preparations are more effective in calling forth antihormones than are purified hormones. The following data are available:

There is ample evidence quoted earlier which indicates that the antihormone accumulates in sufficient quantity to induce the refractory state (96, 99) within two to four weeks after injections are started. Indeed, Hertz and Kranes (97) observed a regression of bovine thyrotropic activity in rabbits after 7 to 12 days of injections. Hamburger (87), using rabbits, observed that an antihormone to the mare serum gonadotropic hormone could be detected as early as the 9th or 10th day after treatment was begun. The time depends apparently upon the species and nature of the animal injected and the amount and quality of the preparation injected.

Simonnet (191) was able with one injection of rabbit antiserum to A.P.L. to protect a rabbit from the action of this hormone for as long as 3 weeks. When the hormone was administered first the antiserum would still check the hormone action if given within 24 hours. Rowlands (172, 173), who has made extensive observations of specificity of antihormones studied the properties of sera of rabbits injected with bovine pituitaries, and observed that these sera inactivated the luteinizing effect of gelding pituitaries. This particular power of the serum increased up to 7 weeks, was then constant for 2 weeks, and thereafter declined somewhat, although the same injections were continued in the donor rabbits. Rowlands and Spence (176), who produced antihormone to pregnant mares' serum gonadotropic hormone in boys, found that the titer in the serum of two patients rose for 3 weeks after the treatment was discontinued. Meyer and Gustus (135) daily injected immature monkeys with 5 R. U. of the gonadotropic substance of pregnant mares' serum; the antihormone was detectable as early as the 27th day, was highly potent at the 39th day, and in some animals persisted 67 days after the injections were stopped.

Zondek and Sulman (221) observed that while the antihormone in rabbit serum had a definite inhibitory effect upon A.P.L. when the two substances were injected separately into the test-animals, yet the inhibitory effect was stronger if the substances were allowed to interact

by mixing and incubating them before the injection. The anti-gonadotropic effect persisted for 8 days after the injection of a single dose. The antihormone circulated in the blood of the test-animals for at least 60 hours after a subcutaneous injection. Eitel and Loeser (55), studying the thyrotropic antihormone in sheep, observed that the titer of the antihormone increased to a maximum at the 4th to 5th week, and then declined, vanishing after 9 to 12 weeks despite continued treatment. The formation of gonadotropic antihormone is said by Zeldenrust (216) to be equally efficient in male or in female rabbits. Mature rats are said to produce more antihormone in response to A.P.L. than do immature rats (78). Ascorbic acid treatment does not enhance the production of antihormones (Zeldenrust, 217). The use of crude extracts (Rowlands and Young, 177) or of glands which have been stored for a period of time (Cutting *et al.*, 35) seems to increase the antihormone-producing power.

*5. Search for antihormones in sera of untreated animals.* The sera of certain untreated animals have an inhibitory effect as if there were antihormones present. This antihormone-like action is perhaps more apt to be effective *versus* the thyrotropic than *versus* the gonadotropic hormone. The thyrotropic inhibitory effect is commonly found in the sera of sheep, but also it is found without any pattern of regularity in the sera of other animals. We do not know enough about these particular inhibitory principles to say whether or not they are the same as antihormones. The fact that normal sera have this inhibitory action has been cited as an argument in favor of the chalone concept, but so far as the writer is aware, the possible rôle of the heterophile antigen (162) in this respect remains to be investigated.

Collip and Anderson (31b) found gonadotropic inhibitory substances in the blood of ten normal patients, but were unable to demonstrate the presence of thyrotropic anti-factors in normal rats when hypophysectomized rats were used as test animals; likewise, they did not find such a principle in normal horse serum. Rowlands and Parkes (175a) found no anti-thyrotropic principle in the sera of normal cows, rabbits, or goats, but it was present to a slight degree in normal horse and sheep sera. Loeser and his associates (54, 55, 125) have found a thyrotropic inhibitory principle in normal ovine and canine blood, and Herold (95) has found a similar principle in guinea pig blood. When the inhibitory activity was present in the serum of untreated sheep Eitel and Loeser (53) were able to increase its titer by the injection of thyrotropic hormone.

Laroche and Simonnet (106) have observed that certain normal human sera contained an inhibitory substance for gonadotropic hormone and also an antibody (precipitin) to A.P.L. Scowen and Spence (186) found a slight antithyrotropic effect in serum of normal rabbits and in normal human serum, but no antithyrotropic effect was found in the serum from two patients with Graves' disease. Zondek and Sulman (223) were not able to find an antihormone of A.P.L. in the blood of pregnant women, nor in the blood of normal or amenorrheic or sterile women, nor in the blood of those suffering from tumors. Sulman (197a) found neither A.P.L. complement-fixing antibodies nor antihormone action in the blood of pregnant women in the 2nd and 4th months of pregnancy and during the first day or the first month postpartum.

Thompson (202b) has observed a thyrotropic inhibitory principle in the blood of two normal ewe lambs. This was not present in the sera of one normal mare, nor in six normal dogs. However, gonadotropic and thyrotropic inhibitory principles were observed in the serum of one human patient with the Cushing syndrome (Case: La Frank) but were not present in the sera of two supposedly normal patients (51). Other inhibitory effects have been obtained with sera from patients with carcinoma (165a, b) and with amenorrhea (198). Picado and Rotter (156, 157, 161) claim that normal human blood, especially of elderly or goitrous people, may have antithyrotropic actions.

Chou (23) stated that no inhibitory effect was found in the sera of five of seven guinea pigs that received normal rabbit serum, whereas the sera of two so treated showed a slight inhibitory action. He quoted Chen (22) of the same laboratory who had observed that the gonadotropic effect of pituitary in an immature rat was lessened hy simultaneous injections of normal rabbit serum. They ascribed this antagonistic action of the serum to its toxic effect, which was manifested by a retarded growth-rate of the animals.

*6. Augmenting effects of sera.* The sera of animals that are undergoing treatment with pituitary extracts sometimes develop the power in test-animals to augment instead of to inhibit the action of gonadotropic extracts. This augmenting effect, sometimes called "progonadotropic", is entirely different from that produced by certain chemicals which act by altering the solubility of the hormone. The former is effective when the serum and hormone interact *in vivo,* whereas the latter must be mixed with the hormonal extracts *in vitro* before the injection. There is no exact knowledge of the nature of the augmenting principle, which has been observed with respect to only the gonadotropic hormone. It

appears to accompany the globulins of the serum. It might be a gonadotropic hormone retained in the animal sera, or another type of substance, possibly an antihormone to the antagonist. This problem deserves more investigation. The available data are listed as follows:

The augmenting principle has been produced in sera by injecting: 1, sheep pituitary extract into dogs or horses (202a); 2, sheep pituitary extract into sheep (30, 170a); 3, swine pituitary extract into a goat (170b); 4, horse pituitary extract or ovariectomized women's urine into rabbits (192). Sulman and Hochman (197b) observed the augmenting effect only once in all their experiments; in this one case the serum was obtained by the daily injection of a rabbit with 2 rat pituitaries for 5 months.

Katzman *et al.* (101a) and Thompson (202a) observed that the augmenting principle in serum stimulates the ovary of the immature rat. This naturally meant that the principle was not species-specific, and more important, it may have been a pituitary principle (110), possibly a follicle-stimulating hormone present in the tested serum. Thompson attempted to rule this out by observing the action of the augmenting principle in hypophysectomized immature rats. He found that it had no effect in these test-animals, whereas it stimulated the ovaries of normal immature rats. This point deserves further study by other investigators, for it appears to be an important method of differentiation. Thompson offered the tentative suggestion that the augmenting principle may be an antihormone to the antagonist (63, 86, 111, 112). Although Rowlands (170a) was inclined to agree with this interpretation, further information must be obtained to clarify the status of this principle, which should be regarded as a retained gonadotropic fraction until proved otherwise.

The augmenting principle is contained in the globulin fraction (202a), as are the antihormones and immune bodies and certain pituitary hormones. Katzman *et al.* (101b) observed that the principle was readily adsorbed by "Willstätter's type A" alumina from a globulin fraction but not from whole serum. It did not dialyze through a Visking membrane. The globulin precipitate could be frozen, desiccated, and then stored for long periods without loss of this activity.

There are other substances that have the power to augment the action of gonadotropic extracts. They are different from the above described principle because they must be mixed with the gonadotropic extract *before* injection, whereas the principle described above has pronounced effects when the mixture is *in vivo*. Examples of the sub-

stances requiring preliminary admixture with the extract for their enhancing properties are: 1, certain substances such as tannic acid (194) and the ions of copper and zinc (65) which produce relatively slowly acting proteinates of the hormonal preparation, and 2, certain elements of whole blood, namely, hemolyzed erythrocytes, hemoglobin, and heme; porphyrin, globin, and ferrous or ferric chloride do not have this effect (McShan and Meyer, 134).

7. *Antithyroid principles.* There are reports of experiments that deal with principles in blood serum, which, following oral administration to animals, inactivate the thyroid and its hormone. Although this particular subject-matter may not be directly within the scope of this review, it deserves brief mention in order to avoid a confusion of these principles with the antihormones. The anti-thyroid principles apparently were discovered in the following manner: The concept of the function of the thyroid gland current in the eighties and nineties of the last century, before the advent of our present knowledge of the physiological actions of the thyroid and its hormone, was that of Schiff and of Horsley, who taught that poisons circulating in the blood were detoxified in the thyroid, and that thyroglobulin represented tho iodized, detoxified product. This idea was somewhat modified when the cause of parathyroid tetany was discovered, but a number of investigators continued to believe that a circulating poison is neutralized by the thyroid.

Ballet and Enriquez (10) apparently were the first to attempt to utilize this inhibitory or "toxic" action of the blood for the treatment of Basedow's disease. They fed the serum of thyroidectomized dogs to thyrotoxic patients and claimed beneficial effects. Likewise, Burghart and Blumenthal (21) fed Basedow-patients the serum from myxedema patients, observing equally good results. Möbius (139) had the Merck pharmaceutical house prepare blood from thyroidectomized sheep, called it "Antithyreoidin-Möbius" (140), and he claimed it was beneficial for Basedow's disease. The material was effective only when fed, not when injected. There were many variations of this sort of treatment, including the feeding of milk from thyroidectomized animals.

This concept of antithyroid substances received further elaboration by Blum (16) who called the inhibitory principle "Katechin". He claimed that all normal blood has an antagonistic effect toward thyroid. He showed that the blood of normal or of thyroidectomized sheep prevents in guinea pigs the loss of weight produced by thyroid feeding. Romeis (166a, b) in 1923 showed that "Katechin" prevents the effect

of thyroid on tadpoles; 5 cc. of blood from a thyroidectomized or a normal sheep neutralized 10 gamma of thyroxin in tadpoles. He claimed that red blood cells, serum, and plasma contain "Katechin." Blum (16) stated that these facts were confirmed by Geszner, who found that diiodotyrosine also was neutralized by "Katechin." Although Anselmino and Hoffmann (5) and others found the "Katechin" in an ether-soluble fraction, Blum (16) said the serum still retained its "Katechin" after extraction with ether, that it was water-soluble, heat-stable, and not (or slowly) dialyzable. Blum (16) stated that an ether-extract of blood did not have this effect, and that the effect was not due to cholesterol.

The reports of Anselmino and Hoffmann (5) on "Katechin" are summarized as follows: The active substance was ether-soluble, and was contained in the fatty acid fraction. The following tissues were rich in "Katechin": fetal blood and liver; adult thymus, ovaries, and bone marrow. Normal blood contained less than the above tissues, but the blood in pregnancy and in Graves' disease was deficient in "Katechin." This substance protected the liver from the loss in glycogen caused by thyroid feeding or by the injection of thyrotropic hormone. It caused an increase in the liver glycogen of normal animals and this was not lost during hunger. The substance did not lower the basal metabolic rate of normal animals. Further contributions on this type of anti-thyroid substance have been made by Asimoff (7), by Gürber and Gessner (84), by Lewit, Berland and Rywkin (115), and others (17, 20).

Abelin (2) also has reported on an anti-thyroid substance which may not be the same as the above, but is now mentioned to show the many facets of this problem. From desiccated thyroid tissue Abelin extracted a substance which he said was an iodized peptone, whose active principle was diiodotyrosine. The feeding of this substance to animals caused an amelioration of the symptoms that normally are produced by thyroid feeding. The substance caused the rapid metamorphosis of tadpoles. One half of the number of a group of mice were protected against the toxic action of acetonitrile. When given together with diiodothyroxin it weakened the character of the resultant hyperthyroidism. Lugol's solution did not have any of the above actions in these experiments. Oehme (143) has stated that glycine, which he says has little effect on the metabolic rate, also has an anti-thyroid action. Schäfer (180) has described an antithyroid effect of vitamin C. Another example of the anti-thyroid effect, which calls

to mind the results of Anselmino and Hoffmann, is the finding of Zain (215) that the feeding of unsaturated fatty acids protected rats against the action of thyroid.

Whatever be the nature of the anti-thyroid substances, so briefly mentioned above, they appear on at least two counts to be different from the antihormones, namely: 1, they are active when administered *per os* while the antihormones are not; 2, they probably are not proteins, while the antihormones appear to be globulins.

*Antibody formation with thyroid preparations.* For the sake of completeness, and for comparison with the data presented on antihormones, some data relative to antibodies for thyroid preparations will be presented. It will be noted that the alluring characteristic of antihormones is their property to inactivate the physiological effects of certain hormones. In this respect the majority of the results with antisera to thyroid preparations differ from those with the antihormones (184). In the review of antihormones by Collip, Selye and Thomson (32a) there is a summary of the formation of antibodies to thyroid preparations that reads as follows:

> . . . The question whether a true immunity with antibody formation may occur as a defence against thyroid extracts was first investigated by Papazolu (1911). This author obtained a positive complement fixation reaction with the serum of patients suffering from Graves' disease against extracts of the thyroids of such patients. Vallagussa (1910) and Kolle (1909) were unable, however, to demonstrate any antibody or antigen in the blood or thyroid of patients suffering from Graves' disease. Ballner *et al.* (1912) found that sera of individuals suffering from goitre, and even sera of non-goitrous individuals, living in the Tyrol where goitre is endemic, give a complement fixation reaction with alcoholic extracts of thyroid tissue. Bauer *et al.* (1937), Bauer (1937), Bauer and Kunewalder (1937) and Bauer and Schächter (1936) found that rabbits chronically treated with thyroxine become resistant to the hormone, and at the same time their serum acquires the property of giving a positive complement fixation reaction with thyroxine or diiodotyrosine. However, they found that this resistance cannot be transferred passively by injecting the serum of a thyroxine-resistant animal into a non-pretreated one. These authors observed, furthermore, that the complement fixation reaction with diiodotyrosine, which is usually positive in cases of severe Graves' disease, became negative following the removal of the hyperactive thyroid. Kestner (1937) reported experiments showing that the serum of sheep chronically injected with pig thyroid emulsions exerts antithyroid effects, in so far as rabbits, dogs and rats treated with this serum show a decrease in metabolic rate. The author believes this to be due to the formation of organ-specific antibodies.
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> The positive results of immunizing experiments against thyroglobulin (Hektoen *et al.*, 1927; and others) have already been mentioned in the historical introduction and need not be discussed here in detail, since there is little evidence of their having anything to do with antihormones in the true sense of the word, especially

since it was shown that *in vivo* antithyroglobulin fails to antagonize the physiological actions of thyroglobulin (Schulhof, 1930). Rosen and Marine (1937) showed that prolonged injections of iodothyroglobulin do not cause refractoriness to the metabolic action of this substance in the rabbit, in spite of the definite immune response (precipitin formation). It also suffices simply to mention the finding of Picado and Rotter (1936), who obtained specific precipitins against thyroid protein.

THE CHEMICAL PROPERTIES OF ANTIHORMONES. The antihormones appear to be proteins, or are closely linked to them. The active principles reside in the globulin fraction of the serum. This fraction contains the immune bodies, and also certain pituitary and other hormones. The antihormone-containing globulin fraction can be precipitated from other constituents of the blood by methods customary for the preparation of immune globulins. The aqueous solutions slowly lose their activity on standing, but the dried preparations retain antihormone activity for long periods of time; likewise, the aqueous solutions are thermolabile. There is some evidence available to show that in the case of A.P.L. the antihormone-hormone linkage may be broken by simple chemical methods. More exact knowledge of the chemical nature of antihormones awaits studies of more highly purified homogeneous preparations of both the hormones and the antihormones.

The literature is represented by the following: There is general agreement that antihormone activity with respect to pituitary gonadotropic and thyrotropic and to adrenal cortical hormones resides in the globulin fraction of the serum (Harington and Rowlands, 88; Hartman *et al.*, 89b; Thompson, 202a; Zondek *et al.*, 219). Some of the nonspecific antibodies that are not antihormones have been precipitated from the globulin fraction by suitable immunological techniques (Gegerson *et al.*, 73). The desiccated globulin fraction retained its antihormone activity for long periods, and the dried product has been washed with acetone or with ether without significantly altering its potency (Zondek *et al.*, 219, 224). In serum the activity is slowly lost on standing even when sterile and refrigerated, but not when frozen. Collip and Anderson (31a, b) state that the thyrotropic antihormone is completely destroyed by boiling at pH 5 for 3 minutes. The thyrotropic and gonadotropic antihormones apparently do not pass through collodion membranes (Harington and Rowlands, 88). Loeser and Trikojus (126) have described the preparation of thyrotropic antihormone by precipitation with benzoic acid followed by elution of the antihormone from the precipitate.

A number of chemical properties of rabbit A.P.L. antihormone were

investigated by Zondek *et al.*, (221), and the following conclusions were drawn: heating for one hour at 70°C. did not destroy the antihormone activity, which, however, was destroyed by heating to 80°C. and by being brought once to the boiling point in a dilute solution. On the other hand, the acetone-dried powder of an active fraction of antiserum survived dry heating for one hour at 100°C. The antihormone was destroyed by digestion with pepsin, trypsin, and N/10 NaOH. It was not affected by treatment with N/50 $NH_4OH$, N/10 HCl, 1 per cent hydrogen peroxide solution, nor by ultra-violet irradiation. It was soluble in 40 per cent acetone but not in 50 per cent. It would not dialyze through cellophane or cuprophane membranes. Further experiments led Zondek and Sulman (222) to conclude that the inactivation of A.P.L. by the antiserum was a reversible process, because they apparently were able with exposure to N/10 NaOH to destroy the inhibitory activity in the A.P.L.-antiserum complex, and to release the antihormone from the complex by destruction of the hormone with N/10 HCl. They found the antihormone was contained in the pseudoglobulin fraction of the serum. On the basis of these observations they concluded that the antihormone resembles an immune body, but has certain unusual properties.

Zondek, Sulman and Hochman (225) observed that antihormone neutralizes A.P.L. quantitatively rather than by a process of adsorption, a quality which they say distinguishes this substance from known groups of immune bodies. The same observers elaborated further methods for the preparation of the antihormone to A.P.L., especially studying various methods for its partial purification. They found that the antihormone was precipitated by many of the common protein precipitants, but that the methods of most practical value were precipitations with acetone, or with saturated ammonium sulphate at the isoelectric point of the diluted antiserum. A combination of these methods gave the most highly purified preparations. Antiserum from rabbits appeared to be more suitable for these studies than antiserum from goats.

THE EFFECTS OF ANTIHORMONES IN ANIMALS. Whatever uncertainty there may be in our minds about the chemical and physical nature of the antihormones, there can be no question of the fact that they have physiological actions. This property has called forth the major portion of the experimental data herein reviewed. The physiological effects of antihormones at present are studied by two chief methods, which are: to observe the effects of the refractory state upon the injected animal

itself, and to compare in test-animals these effects with those produced by injections of the serum from the refractory animal. In order to accomplish physiological changes, other than merely to neutralize an injected extract, the antihormone must be either non-species-specific or at least specific for the species of animal used in the test. In order to interpret the changes resulting from treatment with antihormones, some evidence should be submitted to show that the antiserum used was tolerated by the test-animal without ill effects that could be ascribed to anything other than the antihormone. This last condition sometimes has been as difficult to fulfill with antihormone sera as it has been with hormone preparations.

We do not know exactly how the antihormones achieve their effects, but what data we have suggest that the physico-chemical reactions between the hormone and its specific antihormone occur while they both are in the circulating blood stream. The hormone effectively and rapidly is rendered inactive; its later fate is not known. The antihormones to the pituitary hormones have no direct effect on the subsidiary ductless glands, but there is evidence that they may cause some preliminary hyperactivity of the pituitary itself, and this may, if the action is prolonged, lead to exhaustion atrophic effects in this gland. The effect of the antihormone, then, in addition to neutralizing a coincidentally injected extract, is also to inactivate hormones while they are *en route* in the animal itself from pituitary to subsidiary ductless glands. This creates with respect to the antihormones injected a sort of therapeutic selective hypophysectomy. An atrophy of the appropriate subsidiary glands subsequently occurs, as if hypophysectomy had been performed, and the final physiological effects observed are the results of these atrophies. The more detailed account follows:

*Effects of gonadotropic antihormone.* Rowlands (169, 170b) together with his associates (150, 175a, b, 177) have reported possibly the most complete experiments on the actions of antihormones. A non-species-specific antihormone in rabbit serum had the following effects: 1, inhibited ovulation and corpus luteum formation in the donor rabbits; 2, caused atrophy of the reproductive organs of the adult male rat, and this occurred in the same time-interval that it does after castration or hypophysectomy; 3, suppressed ovulation in the pubertal rat; 4, inhibited implantation of the blastocyst in the rabbit, or caused resorption of the fetuses if injections were begun late in pregnancy. This serum had no effect on the lactation of rats. The foregoing experiments and those of Thompson and Cushing (203b) and Thompson (202c) supple-

ment each other. In these latter experiments a non-species-specific canine antihormone had the following effects: 1, the development of the reproductive system of young rats was held in abeyance during the course of the injections; 2, the pregnancies of dogs were terminated within 100 hours after the first injection, without any evident directly harmful effects on the fetuses.

In their first reports, Collip and his associates (9, 188a, d, e), had shown there was an atrophy of the gonads of the refractory animals; in some cases there were "wheel cells" present in the theca (32c), a finding which is said to be a sign of complete absence of hypophyseal gonadotropic stimulation (188b, c). Failures (226) (and there have been some) to achieve physiological effects in such situations as the foregoing probably have been due to a lack of specificity of the antihormone in the test-animal.

On the anterior pituitary gland the pituitary antihormones have pronounced functional (64, 105) and histopathological effects, and for the convenience of the reader all of the data on this subject will now be given, although some data have to do also with thyrotropic and adrenotropic and possibly other antihormones. The first of the histopathological data comes from the early work of Koyano (104), 1923, whose cytological description of pituitaries affected by an antihormone preparation is remarkably like the later and more complete one of Severinghaus and Thompson (189a, b), who found that the pituitary glands of long-time refractory dogs had all the changes characteristic of castration, of thyroidectomy and possibly of adrenalectomy.

Although numerous reports had been made of "signet-ring" or "castration changes" having been produced in the anterior lobe basophils by antihormones, it appears that Severinghaus (189a, b), the cytologist, was the first to coördinate the observed changes with the new knowledge of pituitary cytology in endocrine disorders. In this particular investigation, the following 3 types of pituitaries were observed: the first were from 2 sheep that had long been injected with a sheep pituitary extract that did not call forth antihormones; the second were from 2 dogs that had developed non-species-specific antihormones after prolonged injections of the sheep pituitary extract; and the third were from 2 dogs injected for 30 days with canine antihormone serum similar to that which might have been obtained from dogs of the second group. Briefly stated, the results showed that the pituitaries of the sheep (without antihormones) showed the characteristic effects of estrogenic hormones, due undoubtedly to the active ovaries of the animals; the

pituitaries of the refractory dogs showed advanced mixed effects, some basophil cells being characteristic of thyroidectomy, and others of castration, while at the same time there was degranulation of the acidophils; the basophils in the pituitaries of the antihormone-injected dogs showed the changes described by Crooke. This change, a characteristic hyalinization of the cytoplasm of the basophils, is found in practically every case within the Cushing syndrome (51).

*Effects of thyrotropic antihormone.* Anderson and Collip (3) observed that this antihormone causes the basal metabolic rate of rats to decrease below the normal level, and the effect of thyrotropic hormone on the metabolic rate to be inhibited. Before the phase of refractoriness actually is apparent the liver regains its glycogen content which had disappeared following the original action of the thyrotropic hormone (Fischbach and Terbrüggen, 66). Loeser (122) has shown that the thyrotropic antihormone prevents the liver-glycogen depleting effect of thyrotropic hormone. The normal thyroid gland of the guinea pig is said to become atrophic after treatment with the antihormone (54). Anderson and Collip (3), and Eitel and Loeser (55) have found that the thyrotropic hormone content of the hypophysis is reduced following treatment with the antihormone.

*Other antihormone effects.* The actions of antihormones to prolactin, to corticosterone, and to the diabetogenic hormone have been described in earlier sections to which the reader is referred. Certain unusual antagonistic effects ascribed to antihormones might be mentioned, such as the following: Russell (178) has reported on the production of refractoriness to the action of anterior pituitary extracts in depressing oxidation of fed carbohydrate. Rodewald (165a, b) claims that in serum from patients suffering with cancer there may be an antihormone having the property to neutralize the action of A.P.L., and also another which inactivates the melanophore principle. Durupt *et al.* (45) have reported on the presence in the urine from certain women of a substance which are antagonistic to estrin. There is not sufficient data presented to indicate the nature of the inhibitory effect. Also reported (183) is a property of serum of pregnant women said to inactivate vasopressin in test-animals.

Here it may be of interest to note that all efforts by the injection of pituitary extracts to reproduce the Cushing syndrome have failed because refractoriness to the adrenotropic hormone has developed in the animals. The adrenotropic factor is believed by some to be overproduced in patients with basophilism, especially in the cases with

basopbilic adenomas and also in the cases where there is no tumor of any gland.

An interesting animal prepared by Thompson and Cushing (203a) perhaps deserves description. Unusual anatomical changes were produced in a fox terrier puppy that was injected for four months with large doses of a crude, ovine pituitary extract. The animal became adipose; bad a strikingly sub-normal metabolic rate as compared to the control; bad a retardation in the osseous development witb incarceration of the teeth and the development of prognathism; the liver became tremendously enlarged, with glycogen deposits of an extent seen only in von Giercke's disease; the skull was eburnated and had the changes which usually follow hypopbysectomy. In some respects the effects of tbe injections were similar to the findings in basophilism, but in others they resembled hypopbysectomy, for the subsidiary ductless glands were strikingly atrophic.

The significance of antihormones. The antihormones are an important class of substances because of their physiological properties which induce the refractory state and alter certain functions of the ductless glands. Their discovery added to our knowledge by introducing a new concept and by further revealing the breadth of an animal's protective reactions to unusual conditions. When they were first described tbe importance of antihormones was recognized by physicians (47, 48) who appreciated not only the possible dangers whicb might result in patients from the induction of the refractory state but also the value should their actions receive successful therapeutic application in certain endocrine disorders.

Most of the hormones now availablo for therapeutic use must be administered parenterally. The induction of the refractory state in a patient obviously must be avoided by using for the therapy non-antigenic preparations. These are homologous preparations, or those of sufficient purity that they have no antihormone-producing power. The pharmaceutical bouses sbould without delay seek metbods to remove the antigenic qualities of tbeir endocrine products intended for parenteral use.

The successful therapeutic use of antihormones in man has yet to be reported. A number of difficulties have stood in the way of those who have attempted to test their actions in man. The toxic factors present in many animal sera must be removed before the preparation may be tested (100). The antihormone must be specific not only for the hormone but also for tbe species, or it must be completely non-

species-specific, able to accomplish the same effect as a specific antihormone.

It is not easy to prepare large quantities of a suitable specific serum, for when the antihormone potency is sufficiently great the non-species-specific effects on the injected animal are such that bleeding in any quantity is not well tolerated, and the animal also becomes susceptible to other ailments. Despite these difficulties, which may be overcome, there is need for tests of the actions of antihormones to be made, with suitable precautions for safety, in man. Valuable physiological data will be obtained no matter what may be the physiological action as judged from the therapeutic standpoint. One cannot predict the effects to be observed in man following the administration of properly prepared specific antihormones, for knowledge of the hormonal balance in hyperthyroidism, in pregnancy, and in other states in which antihormones might be tested is still incomplete. Conceivably the endocrinologist may utilize the action of specific antihormones in the study of the interrelations of the endocrine glands.

The study of antihormones is founded on a broad base, and like so many problems of the present this requires the application of a number of techniques. There is great need for well planned investigations of antihormones by the immunologists, who should have the close collaboration of endocrinologists, for the serological and biological tests must be parallel. What the investigators need for this study are highly purified and homogeneous preparations of the hormones and antihormones.

*In conclusion* one may state that the antihormones now appear to be immune bodies of unusual type. These substances have important properties whereby they inactivate hormones and thus indirectly produce changes in the ductless gland physiology. Although in some circumstances they are unwanted "artifacts" or complicating factors, their physiological actions conceivably may find some useful application.

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